



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/11	A2	(11) International Publication Number: WO 98/14567 (43) International Publication Date: 9 April 1998 (09.04.98)
(21) International Application Number: PCT/US97/18094 (22) International Filing Date: 30 September 1997 (30.09.97) (30) Priority Data: 60/027,729 1 October 1996 (01.10.96) US (71) Applicant (for all designated States except US): ADVANCED RESEARCH & TECHNOLOGY INSTITUTE [US/US]; 501 North Morton Street, Bloomington, IN 47404 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MARTIN, William, J. [US/US]; 7556 Chablis Circle, Indianapolis, IN 46278 (US). WISNIOWSKI, Paul [US/US]; 5914 Petersburg Parkway, Indianapolis, IN 46202 (US). (74) Agent: HIGHLANDER, Steven, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING BACTERIAL GROWTH (57) Abstract <p>The present invention relates generally to the field of bacteriology. More particularly, it concerns methods and compositions for the treatment of bacterial infection employing oligonucleotides targeted to the Shine-Dalgarno region of prokaryotes to inhibit bacterial protein expression and hence inhibit bacterial infection.</p> <p style="text-align: right;">BEST AVAILABLE COPY</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

DESCRIPTION

METHODS AND COMPOSITIONS FOR INHIBITING BACTERIAL GROWTH

BACKGROUND OF THE INVENTION

The present application is related to U.S. Patent Provisional Application Serial No. 60/027,729, filed October 1, 1996. The entire text of the above-referenced disclosure is specifically incorporated by reference herein without disclaimer.

1. Field of the Invention

The present invention relates generally to the fields of bacteriology. More particularly, it concerns methods and compositions for the treatment of bacterial infection employing novel oligonucleotides as antimicrobial agents.

2. Description of Related Art

Lung infection is the leading cause of morbidity and mortality in a number of diseases. *Pseudomonas aeruginosa* is an opportunistic pathogen that may infect virtually any tissue. Infection is facilitated by the presence of an underlying diseases and is particularly prevalent in cystic fibrosis and certain cancers patients. *P. aeruginosa* is typically an extracellular pathogen and therapeutic strategies to eradicate *P. aeruginosa* may do so by directly exposing the bacteria to the antimicrobial substance. In contrast *Mycobacterium tuberculosis* is an extracellular pathogen and alveolar macrophage is the host within the lung for the microorganism. It is estimated that as few as 2 to 3 *Mycobacterium tuberculosis* (TB) organisms can cause pulmonary infection (Canetti, 1991). Organisms are engulfed by alveolar macrophages (AMs) and survive and replicate within the intracellular environment. The mycobacteria are protected within this environment from many adverse host responses. Conventional antibiotic therapy usually kills intracellular mycobacteria; however, the increasing frequency of multidrug resistant (MDR) strains of TB and mycobacteria other than TB (MOTT) is becoming a major clinical problem. For antimycobacterial therapy to be effective, the therapy will need to kill mycobacteria within host cells such as the alveolar macrophage.

In the case of cystic fibrosis the genetic defects underlying the disease are a series of mutations in a protein called cystic fibrosis transmembrane regulator (CFTR) (Collins, 1992). The mutations in the CFTR gene impair chloride and water transport (Jiang *et al.*, 1993) which results in a thick, dry, viscous mucous lining the airways of the lung. Mucin hyper excretion is characteristic of cystic fibrotic lungs. Although the exact mechanisms are speculative at best, patients with cystic fibrosis are susceptible to a series of infections beginning with *Staphylococcus aureus*, followed by *Haemophilus influenzae* then *Pseudomonas aeruginosa*. Currently there are successful therapies for *S. Aureus* and *H. Influenzae*; however, current treatments for *P. aeruginosa* are inadequate and *P. aeruginosa* infections are the leading cause of death in patients with cystic fibrosis (Lloyd-Still and Wessel, 1990; Davis, 1985; Vasil, 1986; Gilligan, 1991).

In addition to causing infections in patients with cystic fibrosis, *P. aeruginosa* is a common pathogen in certain immunosuppressed populations, such as burn and cancer patients, as well as individuals with AIDS (Nichols *et al.*, 1989; Rolston and Bodey, 1992; Mendelson *et al.*, 1994). Since the first report of *P. aeruginosa* infection in 1890, the organism has been increasing associated with bacteremia and currently accounts for 15% of cases of Gram negative bacteremia. The overall mortality associated with such infection is about 50% thereby making *P. aeruginosa* infection one of the most lethal. Although many strains are susceptible to a variety of antibiotics including gentamicin, tobramycin, colistin and amikacin, once established *P aeruginosa* infection is extremely tenacious.

As stated above, one reason for the difficulty in eradication of bacterial infections is the increase in strains resistant to current antibiotics (Neu, 1992), especially aminoglycosides and quinolones. Aminoglycosides bind to the 30S ribosomal subunit and interfere with bacterial protein synthesis (Hutchin and Cortopassi, 1994). The most predominant mechanism of aminoglycoside resistance involve the production of deactivating enzymes (Schwocho *et al.*, 1995). Deactivating enzymes such as acetyl transferase and phosphotransferase are generally carried on plasmid and transposons, quickening the rate of acquired resistance among *P. aeruginosa* populations. Quinolones, on the other hand, inhibit DNA gyrase activity (Hooper and Wolfson, 1989). This inhibition is specifically targeted to the cleavage and ligation activity

of DNA gyrase for double-stranded DNA. This results in DNA strand breakage of the bacterial chromosome, a lethal effect for the pathogen. Quinolone resistance mechanisms include altered DNA gyrases and a decrease in drug permeability through the membrane (Sanders *et al.*, 1984). Increasingly, drug resistant strains are creating a need for new therapeutic approaches in the treatment of bacterial infections.

There is a clear need for methods and compositions for the treatment of Gram-negative bacterial infection that may be used to combat these lethal infections in a wide variety of diseased states.

SUMMARY OF THE INVENTION

The present invention provides novel methods and compositions for the treatment of Gram-negative bacterial infection that may be used alone or in combination with conventional regimens to eradicate potentially lethal infections in a wide variety of diseased states.

Thus, the present invention provides in a specific embodiment, a method for inhibiting bacterial protein expression comprising the steps of providing an oligonucleotide of between about 10 to about 35 consecutive bases of the 3'-end of a bacterial 16S rRNA; and contacting the oligonucleotide with a bacterium, whereby protein expression of the bacterium is inhibited.

In certain embodiments, the oligonucleotide may be modified to include a transport moiety and DNA phosphate modifications to increase nuclease resistance. In other embodiments, the oligonucleotide is formulated in a liposome.

In particular embodiments, the bacterium may be selected from the group consisting of *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Moraxella lacunata*, *Streptomyces scabies*, *C. perfringens*, *E.coli*, *Salmonella typhi*, *Cornebacterium coyleiae*, *Magnetic coccus*, *Azoarcus evansii*, *Sphingomonas trueperi*, *Burkholderia*, and *Chlamydia*.

In certain other embodiments, the transport moiety is selected from the group consisting of an amino acid, biotin, folate and a carbohydrate. In more specific embodiments, the amino acid is selected from the group consisting of arginine, asparagine, glutamine, glycine, valine, isoleucine, leucine, methionine, proline, tryptophan and valine.

5

In other embodiments, of the present invention, the oligonucleotide comprises the sequence CCU/TCC. In yet another embodiment, the oligonucleotide comprises at least one phosphoramidite residue.

10

In those embodiments wherein the oligonucleotide is formulated within a liposome, the liposome further comprises a bacterial targeting moiety.

15

Also contemplated by the present invention is a method for treating a bacterial infection in a patient comprising the steps of: providing a liposomal formulation comprising an oligonucleotide of between about 10 to about 35 consecutive bases of the 3'-end of a bacterial 16S rRNA in a pharmaceutically acceptable form; and administering the liposomal formulation to the patient. In particular embodiments, the method further comprises administering to the patient an antibiotic. In particular embodiments, the antibiotic may be a sulfonamide, a quinolone, a penicillin, a cephalosporin, a beta-lactam antibiotic, an aminoglycoside, or a tetracycline.

20

25

An oligonucleotide of between about 10 to about 35 consecutive bases of the 3'-end of a bacterial 16S rRNA is also provided by the present invention. In particular embodiments the 16S rRNA is derived from *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Moraxella lacunata*, *Streptomyces scabies*, *C. perfringens*, *E.coli*, *Salmonella typhi*, *Cornebacterium coyleiae*, *Magnetic coccus*, *Azoarcus evansii*, *Sphingomonas trueperi* *Burkholderia*, or *Chlamydia*.

30

In specific embodiments the oligonucleotide is about 15 bases in length. In other embodiment the oligonucleotide is about 20 bases in length. In still further embodiments, the oligonucleotide is about 25 bases in length. In other embodiments, the oligonucleotide is about 30 bases in length. In more particular embodiments the oligonucleotide is selected from the

group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12.

Also contemplated by the present invention is a liposomal formulation comprising an oligonucleotide of between about 10 to about 35 consecutive bases of the 3'-end of a bacterial 16S rRNA. In certain embodiment the liposomes of the formulation include multilamellar vesicles. In particular embodiments the liposomes comprise at least one of the lipids selected from the group consisting of DMPC, DCP, DMPG and cholesterol. In other embodiments, the liposomes of the formulation further comprise a bacterial targeting moiety.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A and FIG. 1B: FIG 1A Illustrates the ability of the competitive oligonucleotide to inhibit growth of *M. avium*. FIG. 1B. *M. avium* growth was maintained in the presence of the "nonsense" oligonucleotide but was greatly decreased in the presence of the Shine-Dalgarno (SD) oligonucleotide.

FIG. 2A and FIG. 2B. The effect of biotinylation of the oligonucleotide. FIG. 2A demonstrates that the biotinylated form is more readily taken up by *M. avium* as compared to

the non-biotinylated form. FIG. 2B biotinylated oligonucleotide has a greater inhibitory effect on the growth of *M. avium* as compared to the non-biotinylated form.

FIG. 3. 3'-FITC-labeled phosphorothioate oligonucleotide (50 μ M) with and without a 5'-alanine was incubated overnight with *P. aeruginosa*. The next day *P. aeruginosa* organisms were washed $\times 2$ with 500 μ l saline solution. The organisms were resuspended in 200 μ l of saline solution and transferred to a microtiter plate. Transport was measured by fluorimetry. Each sample was done in triplicate.

FIG. 4. Effect of 5'-alanine phosphorothioate SD on the growth of *P. aeruginosa*.

FIG. 5. Effect of varying concentrations of 5'-alanine phosphorothioate SD oligonucleotide targeted to *P. aeruginosa* 16S rRNA 3'-end on the growth of *P. aeruginosa*.

FIG. 6A and FIG. 6B. FIG. 6A The data indicate that *Pseudomonas* attachment can easily be quantified and increases as a function of time with an apparent plateau between 4 and 6 h. FIG 6B. The data indicate that modification of the oligonucleotide with a 5'-alanine significantly enhances the inhibitory effect of the oligonucleotide on *P. aeruginosa* attachment suggesting that synthetic oligonucleotides can be used a tool to link attachment with the production of virulence factors.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Bacterial infections represent a significant challenge in the treatments of a wide variety of disease. Declining public health measures and poor patient compliance in the 1980's and 1990's, have permitted the re-emergence of diseases such as TB, and the additional problem of an increased frequency of MDR strains (Reichman, 1991; Bloom and Murray, 1992; Nardell, 1991; Centers for Diseases Control and Prevention, 1993; Raviglione *et al.*, 1995). Likewise, MDR presents a formidable adversary in the treatment of diseases such as cystic fibrosis, many cancers and AIDS, where an underlying disease condition allows the infective agent to take hold and MDR prevents effective treatment.

As with many infectious disorders (Johanson *et al.*, 1979), attachment of the microbe to certain host cells is thought to be the initial phase of the infection. In bacterial infection, early colonization is closely associated with the appearance of virulence factors. For example, in *P. aeruginosa*, these virulence factors are excreted to obtain nutrients, impair the immune system (Pedersen and Kharazmi, 1987; Theander *et al.*, 1988; Kharazmi *et al.*, 1984a; 1984b; Kharazmi *et al.*, 1986) and assist in attachment (Saiman *et al.*, 1990; Woods *et al.*, 1981). Current concepts suggest that these virulence factors are directly and/or indirectly responsible for lung damage as the infection progresses. Virulence factors involved in attachment include elastase, phospholipase C and neuraminidase. These factors are believed to alter and/or injure the epithelial cell surface in order to facilitate bacterial attachment (Saiman *et al.*, 1990).

The present inventor has had a long term interest in microbial attachment mechanisms (Wisniowski and Martin II, 1995; Wisniowski *et al.*, 1994; Prottratz and Martin II, 1990; Pottratz *et al.*, 1991; Limper and Martin II, 1990). *Pseudomonas* attachment to the epithelial cell surface is thought to occur by several mechanisms. For example, elastase activity effectively removes fibronectin from the epithelial cell surface and since fibronectin is a barrier to bacterial attachment, elastases excreted by the bacteria increase attachment to the host cell (Azghani *et al.*, 1992). Neuraminidase, on the other hand, is thought to expose binding sites on the surface of epithelial cells by hydrolyzing sialic acid from membrane glycolipids (Cacalano *et al.*, 1992). It appears that the first step in *P. aeruginosa* attachment to the upper airway epithelial cells occurs *via* pili and nonpili adhesins. Apparently, the pili recognize sialic residues of glycolipids on the epithelial cell surface (Baker *et al.*, 1990). Pili proteins are not expressed constitutively and are under environmental control (Prince, 1992). It has been suggested that several genes which include genes for pili and virulence factors are under the same regulatory mechanisms (Saiman *et al.*, 1990). Other mechanisms associated with *P. aeruginosa* attachment include epithelial damage through a previous infection which increase bacterial attachment (Ramphal *et al.*, 1980). *In vivo*, *P. aeruginosa* bind to glycosphingolipids within the mucin as glycoproteins in the mucin show specificity for the bacterium (Carnoy *et al.*, 1994).

Thus, bacterial protein expression plays a crucial role in the initial stages of infection. It is clear that preferential inhibition of bacterial protein expression would be extremely advantageous in combating bacterial infection. The present invention provides methods and

compositions for inhibiting the growth of bacteria. The Shine-Dalgarno sequence of prokaryotic mRNA facilitates bacterial protein expression and such a region is not found in eukaryotic mRNA thus bacterial protein synthesis may be specifically targeted. The compositions of the present invention involve the use of oligonucleotides which bind bacterial ribosomal RNA (rRNA) at the Shine-Dalgarno recognition site, thereby preventing the binding of the mRNA and inhibiting protein synthesis. In this regard, the gene sequences from the 16S rRNAs of many bacteria are known and have been reported in the literature. the skilled artisan is referred to Genbank, which has listings of 16S rRNA from numerous bacterial origins. The 16S RNA sequences for *P. aeruginosa*, *M. avium*, *M. tuberculosis*, *Staphylococcus aureus* and *Burkholderia cepacia* are presented in SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12 by way of example. Other sequences are listed in Genbank and incorporated herein by reference include those for *Moraxella lacunata* (Genbank Accession Number D64049), *Streptomyces scabies* (Genbank Accession Number D63862), *C. perfringens* (Genbank Accession Number Y12669), *E.coli* (Genbank Accession Number Z83204), *Salmonella typhi* (Genbank Accession Number U88545), *Cornebacterium coyleiae* (Genbank Accession Number X96497), *Magnetic coccus* (Genbank Accession Number X61607), *Azoarcus evansii* (Genbank Accession Number X77679), *Sphingomonas trueperi* (Genbank Accession Number X97776), *Burkholderia* (Genbank Accession Number X92188), and *Chlamydia* (Genbank Accession Number D88316).

In order for the oligonucleotide preparation to be effective as a therapeutic agent it is necessary to deliver such a composition to the bacterial infection. Such delivery presents a two-fold challenge. In the first instance, it is necessary to bring the therapeutic composition in contact or proximity with the site of infection and secondly, there is the problem of uptake of the therapeutic composition by the bacteria so that the oligonucleotide may be in a position to bind to the rRNA of the bacteria to halt or abrogate the protein synthesis of that bacteria.

The present invention provides methods and compositions that allow for an effective delivery and uptake of the therapeutic compositions disclosed herein. The inventors have discovered that the derivatization of the oligonucleotide compositions with amino acids, such as for example, alanine, greatly facilitates transport. In another embodiment the present invention derivatizes the oligonucleotide using biotin to achieve a more effective delivery to the target

site. Such compositions may, of course, be used in combination with conventional therapies already in place for the treatment of bacterial infection. Such methods and compositions are described in further detail herein below.

5 The inventors data suggests that an oligonucleotide targeted to 3'-end of the 16S rRNA can dramatically reduce attachment of *P. aeruginosa* to epithelial cells. This reduced attachment may correlate with the reduced production in the above virulence factors. Sublethal dose of antibiotics result in a decrease in the production of virulence products (Grimwood *et al.*, 1989a; 1989b). This decrease correlates with improved condition of the patient suggesting
10 (Woods *et al.*, 1986), that an antimicrobial oligonucleotide will likely have the same effect.

1. **Shine-Dalgarno Region**

Shine and Dalgarno (1974) noted that the 16S RNA contains a seven-base pyrimidine-rich sequence near its 3'-end. Additionally it was observed that bacterial messages contain a
15 complementary purine-rich region (Shine-Dalgarno region) located approximately 10 bases to the 5' side of the AUG initiation sequence. It was therefore suggested that this polypyrimidine region of 16S rRNA might promote the interaction of the 30S subunit with the region of the mRNA containing the initiator AUG. On the basis of these observations, it soon became clear that the degree of pairing determines the avidity of the ribosome for the message and hence the
20 efficiency of translation. The Shine-Dalgarno sequences are variable in length and position relative to the AUG start codon and complementarity varies between 3 and 9 bases. Thus, specific pairing with the Shine-Dalgarno sequence is important in the discrimination by the bacterial ribosomes of the message start signals.

25 The foregoing applies only to prokaryotes and it is important to note that although there is much conservation between the 3'-end of the rRNA of prokaryotes and eukaryotes, the key CCUCC sequence involved in the Shine-Dalgarno interaction is absent from eukaryotic 18S rRNA (Hagenbuchle *et al.*, 1978). Thus, the 3'-end of the 16S rRNA contains an anti-Shine-Dalgarno sequence that serves as a messenger RNA (mRNA) ribosomal binding site for
30 subsequent protein synthesis. The present inventors have designed oligonucleotides to bind to the 3'-end of the rRNA, preventing the binding of the bacterial mRNA and thereby acting as a competitive inhibitor for protein synthesis in bacteria. This anti-Shine-Dalgarno sequence is

not present in the analogous human 18S rRNA, thereby allowing the inventor to develop a pathogen specific therapeutic strategy.

In this regard, the gene sequences from the 16S rRNAs of many bacteria are known and have been reported in the literature. As one illustrative example, the gene sequence for the 16S rRNA has been determined for several mycobacteria including *M. avium* (van der Giessen *et al.*, 1994; SEQ ID NO:9). With the knowledge of the reported 16S rRNA sequence and the appropriate and specific flanking regions for the 16S rRNA (which binds the Shine-Dalgarno sequence on mRNA), the inventors constructed an exemplary competitive oligonucleotide that binds the 3'-end of the 16S rRNA of bacteria.

It is important to determine the correct regions of the RNA base sequence immediately 3' and 5' of the anti-Shine-Dalgarno sequence necessary to design a competitive oligonucleotide. This includes all the base sequence to the 3' of the anti-Shine-Dalgarno and 20-30 base sequences to the 5' of the anti-Shine Dalgarno sequence.

DNA:	3'-CGCCAACCTAGTGGAGGAAAG-5'	-coding strand: SEQ ID NO:1
	5'-GCGGTTGGATCACCTCCTTTC-3'	-anticoding strand;
		SEQ ID NO:2
rRNA	5'-GCGGUUGGAUCACCUCCUUUC-3'	SEQ ID NO:3
competitive oligonucleotide	3'-CGCCAACCTAGTGGAGGAAAG-5'	SEQ ID NO:4

The following schematic illustrates yet another synthetic oligonucleotide and the 3'-end of *P. aeruginosa* 16S rRNA. The upper sequence is the synthetic oligonucleotide targeted for end the 3'-end of *P. aeruginosa* 16S rRNA and will be referred to as the Shine-Dalgarno(SD) oligonucleotide. The oligonucleotide was synthesized by the Cyclone plus DNA synthesizer (Milligen) using phosphoroamidite chemistry. The Shine-Dalgarno sequence is in bold. The lower sequence is the 3'-end of *P. aeruginosa* 16S rRNA (Toschka *et al.*, 1988).

SD phosphorothioate oligonucleotide

3' CGC CGA CCT AGT **GGA GGA** AT 5' SEQ ID NO:5

5' GCG GCT GGA TCA CCT CCT TA 3' SEQ ID NO:6

3'-end of *P. aeruginosa* 16S rRNA

5 In this manner, the competitive oligonucleotide binds that portion of the bacterial ribosome which recognizes the Shine-Dalgarno sequence on bacterial mRNA, thus effectively blocking mRNA translation and protein synthesis in the bacteria. For instance, the inventors have employed the BACTEC assay system to quantify mycobacteria viability from monolayer cell culture (Reddy *et al.*, 1994), and shown the ability of the first designed oligonucleotide (SEQ ID NO:5), which binds the 3'-end of the 16S rRNA, to significantly inhibit growth of *M. avium* and *M. tuberculosis* (SEQ ID NO:9 and SEQ ID NO:10 depict the 16S RNA sequences of these bacteria). In contrast to the SD oligonucleotide for Mycobacterium, *M. avium* growth was maintained in the presence of the "nonsense" oligonucleotide (SEQ ID NO:7).

20 In a particular embodiment, the present invention demonstrates the effects of specific oligonucleotides targeted to the anti-Shine-Dalgarno sequence located at the 3'-end of *P. aeruginosa* 16S rRNA (SEQ ID NO:6). The Shine-Dalgarno sequence AGGAGG is present on prokaryotic mRNAs and is not present on eukaryotic mRNAs. By base pairing with the anti-Shine-Dalgarno sequence and the correct flanking sequences, and therefore, function as a competitive inhibitor for mRNA in only *P. aeruginosa*. Since mRNAs in eukaryotic cells, such as airway epithelial cells, do not contain a Shine-Dalgarno sequence, the oligonucleotide offers the opportunity for specificity for the pathogen alone.

25 As used herein, the term "oligonucleotide" is intended to encompass oligodeoxynucleotides which are naturally-occurring or synthetic oligodeoxynucleotides, or naturally-occurring or synthetic oligoribonucleotides, and can include modified forms thereof which are resistant to nuclease digestion so as to increase their ability to inhibit or prevent protein synthesis in the target bacterial cells (*e.g.*, by blocking the 3' or 5'-end using conventional techniques such as adding a phosphorothioate-linked nucleotide).

As used herein an oligonucleotide is a short length of single-stranded polynucleotide typically up to about 30 residues in length. The Shine-Dalgarno sequences of many bacteria are known making it possible for one of ordinary skill in the art to synthesis an oligonucleotide to mimic such a sequence. The length of the oligonucleotide may vary between 20 and 40 nucleic acids with a 30mer generally being preferred. Of course it is understood that the length may be between about a 10mer and 40mer, between about a 15mer and a 35mer, between about a 20mer and between about a 30mer, a 21mer, a 22mer, a 23mer, a 24mer, a 25mer, a 26mer, a 27mer, a 28mer or a 29mer. Of course, these lengths are only exemplary and any length in-between any two of points is also expected to be of use in the present invention.

Oligonucleotides now can be prepared routinely using oligonucleotide synthesizers without recourse to molecular biology (Gait, 1984). These synthesizers use solid phase phosphoramidite chemistry to synthesize a section of oligonucleotide from 3'-end by successively adding and coupling protected, activated nucleic acids in the form of cyanoethylphosphoramidites to a starting nucleotide that has been anchored to the surface of a glass bead sold support. The efficiency of the coupling at each stage is greater than 99.9% and oligonucleotides of up to a length of 100mer can be produced to over 99% purity levels.

The flanking regions of the oligonucleotides are those regions that are complimentary to the flanking regions of the anti-Shine-Dalgarno sequence of the 16S rRNA.

2. Uptake of the Shine-Dalgarno Sequence

One of the requirements for an effective therapy is that the therapeutic agent be taken up by the bacteria such that it can bind to the Shine-Dalgarno region of the mRNA of that particular organism and thereby inhibit its translation. Previous *in vitro* studies have demonstrated that oligonucleotides targeted to the 3'-end of the *Escherichia coli* 16S rRNA bind to the 30S ribosomal subunit (Trudel *et al.*, 1981; Taniguchi and Weissmann, 1978; Eckhardt and Luhrmann, 1979; Jayaraman *et al.*, 1981). These oligonucleotides varied in length from five to nine nucleotides and contained partial or complete Shine-Dalgarno sequences. These oligonucleotides also inhibited mRNA binding to cell free ribosomes. Further, Jayaraman *et al.* (1981) demonstrated that a seven-base oligonucleotide inhibited *E. coli* protein synthesis in cell free systems and had no effect on eukaryotic protein synthesis.

However, these oligonucleotides were not effective against wild type *E. coli* due to the lack of transport of the oligonucleotide into the bacterium.

Presently the inventors have shown that modifying the oligonucleotide sequence will facilitate transport of the oligonucleotide into the bacterium. Such modifications can include for example, oligonucleotides to which additions have been made to their 3' or 5'-end to increase uptake into the bacterial cell, e.g., by the attachment of amino acids, peptides or other primary amines, or sugars, such as lactose, to the 3' or 5'-end of the oligonucleotide. Such means for regulating the uptake of the oligonucleotides are well known to those skilled in the field and will be readily applied to the present invention. Also included in the present invention are methods of stabilizing the oligonucleotide by for example, synthesizing nuclease resistant monothiooligonucleotides using standard phosphoramidate chemistry and incorporating Beaucage reagent (Iyer *et al.*, 1990) during the oxidation step. In addition to these strategies, agents may be attached to the oligonucleotide to facilitate binding with the target RNA. For instance, acridine can be added to the 3'-end of the oligonucleotide using an acridine CPG solid support column (Glen Research, Sterling, VA).

A) Amino acid modification of the oligonucleotide

The inventor has demonstrated that the amino acid alanine facilitates transport of a synthetic oligonucleotide targeted to the Shine-Dalgarno region *P. aeruginosa* into such organisms. Other amino acids may increase the efficiency of oligonucleotide transport. The synthetic method described herein below allows amino acid derivatives with unprotected side chains be coupled to the 5'-end of an oligonucleotide. Amino acid derivatives with protected side chains require de-protection that would destroy the oligonucleotide. All 20 amino acids will be assayed to facilitate transport.

Generally, for the addition of a 5'-amino acid, a 5'-amino modifier is added during the automated synthesis. The 5'-monomethoxytrityl group is removed manually as suggested by the manufacturer by incubating the solid support with de-blocking reagent, such as 2.5% dichloroacetic acid in dichloromethane for 4 × 5 min. For this and subsequent steps, the solid support is removed from the column and placed in a 1.5 ml siliconized eppendorf tube. After the detritylation step, the solid support is washed two times in N,N-dimethylformamide.

Biotin is added to the oligonucleotide during the automated synthesis using a biotin phosphoramidite precursor.

5 To couple an amino acid onto the 5'-end of the oligonucleotide, an f-moc-L-amino acid dissolved in N,N-dimethylformamide is added to the solid support in a 100-fold molar excess to the oligonucleotide. O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate (HATU) and diisopropylethylamine (DIPEA) is used as coupling agents as the solid support will be incubated with these reagents for 30 min. The f-moc L-amino acid:HATU:DIPEA ratio used during the coupling is about 1:1:2. The oligonucleotide is removed from the solid support through a 90 min incubation in 30% ammonium hydroxide. Further incubation in 30% ammonium hydroxide for a total of 24 h will be required to remove all of the protecting groups from the oligonucleotide and the f-moc protecting group from the amino acid. The amino acid coupled oligonucleotide will be lyophilized to dryness for subsequent purification.

Quality control and purification of the oligonucleotides is performed by reverse phase HPLC with a UV detector. The reverse phase column will be run with a linear gradient of acetonitrile (20%-50%) and 0.1 M triethylammonium acetate in H₂O (80%-50%). A successful synthesis will be seen as a doublet peak on HPLC chromatogram. The doublet results from the R_p and S_p configuration of the phosphorothioate linkages immediate adjacent to the 5'-amino acid group (Stec *et al.*, 1985).

B) Biotinylation

25 To facilitate transport, Low *et al.* (Leamon and Low, 1991; Horn *et al.*, 1992) have modified macromolecules with vitamins such as biotin and folate. Biotin-modified proteins facilitate transport into plant cells while folate modification increases oligonucleotide transport into cancer cells. In a preferred aspect, the oligonucleotide is biotinylated at the 5' end of the oligonucleotide using biotin phosphoramidite precursor (Glen Research, Sterling, VA).

30 As discussed earlier oligonucleotides may be prepared using solid-phase phosphoramidate chemistry well known to those of skill in the art. Other strategies to

biotinylate the oligonucleotide include a functional group at the terminus of the oligomer to be labeled. This group must be inherently reactive toward the labeling reagent and generally tend to be a primary amine or thiol group introduced during the synthesis of the oligonucleotide via an appropriately protected phosphoramidite. The incorporation of protected amines and thiols and their subsequent modifications have been described extensively in the literature and are well known to those of skill in the art (Lin and Prusoff, 1978; Toren et al., 1986; Wachter et al., 1986; Connolly, 1987; Conolly and Rider 1985; Bischoff et al., 1987).

The labeled oligonucleotides may then be purified by HPLC or PAGE. In general, labeled oligomers will migrate more slowly on PAGE than unlabelled control oligomers, unless the label is strongly anionic. When using HPLC a suitable reverse phase column is set up at an appropriate flow rate employing TEAA and acetonitrile. The acetonitrile concentration may be as high as 30% and allows the biotinylated oligonucleotides to elute after the unmodified DNA. Similar conditions may be used to elute oligonucleotides that have been labeled with fluorescein, rhodamine and Texas red simply by altering the organic content of the mobile phase. More specific details of the HPLC purification of biotinylated oligonucleotides is given elsewhere in the specification.

C) Glycosylation

Glycosylation will be achieved using the same strategies to couple amino acids to the 5'-end of the oligonucleotide providing the sugar moiety containing a carboxylic acid group. For instance, glucose could be coupled to the 5'-end of the oligonucleotide via glucuronic acid.

Decreasing Homology between Prokaryotic and Eukaryotic rRNA

Although there is much conservation between the 3'-end of the rRNA of prokaryotes and eukaryotes, the key CCUCC sequence involved in the Shine-Dalgarno interaction is absent from eukaryotic 18S rRNA (Hagenbuchle *et al.*, 1978). Thus, eukaryotic rRNAs do not contain anti-Shine-Dalgarno sequences. There is a 7 base pair mismatch between the proposed SD oligonucleotide and the 3'-end of eukaryotic 18S rRNA. Thus it is unlikely that the SD oligonucleotide will interfere with eukaryotic protein synthesis (Jayaraman *et al.*, 1981). This fact notwithstanding, any chance likelihood of the oligonucleotides of the present invention interfering with eukaryotic protein synthesis may further be eradicated by varying the length of

the oligonucleotide and by the use of spacer phosphoroamidites in regions of homology between the 3'-ends of the *P. aeruginosa* 16S rRNA and human 18S rRNA will be incorporated.

Decreasing the length of the nucleotide will decrease homology to the human 18S rRNA, thereby increasing specificity for the pathogen. The spacer phosphoroamidite is designed to selectively replace nucleotide bases and substitute "blanks" in the oligonucleotide sequence. The chemical name for the spacer phosphoroamidite is 3-O-dimethoxytrityl-propyl-1[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (Glen Research, Sterling, VA). These "blanks" in the oligonucleotide sequence will be inserted where homology exists between the prokaryotic 16S rRNA and the human 18S rRNA, thereby significantly reducing base pairing and homology. This strategy, if necessary, should significantly enhance specificity for the pathogen.

3. Purification of the Modified Oligonucleotides

The present invention employs a number of different purification techniques including those based on electrophoresis and chromatography in order to purify the oligonucleotides of the present invention. Such techniques are described in more detail herein below.

A) Chromatography

In certain embodiments of the invention, it will be desirable to produce functional oligonucleotide or variants thereof. Purification techniques are well known to those of skill in the art. Analytical methods particularly suited to the preparation of a pure oligonucleotide are ion-exchange chromatography; polyacrylamide gel electrophoresis. A particularly efficient method of purifying oligonucleotides is HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an oligonucleotide. The term "purified oligonucleotide" as used herein, is intended to refer to a composition, isolatable from other components of a reaction mixture.

Generally, "purified" will refer to an oligonucleotide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the oligonucleotide forms the major component of the composition, such as constituting about 50% or more of the oligonucleotides in the composition.

Various methods for quantifying the degree of purification of oligonucleotides will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of oligonucleotide within a fraction by PAGE analysis.

It is known that the migration of a molecule can vary, sometimes significantly, with different conditions of PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified oligonucleotides may vary.

Ion-Exchange Chromatography. The basic principle of ion-exchange chromatography is that the affinity of a substance for the exchanger depends on both the electrical properties of the material and the relative affinity of other charged substances in the solvent. Hence, bound material can be eluted by changing the pH, thus altering the charge of the material, or by adding competing materials, of which salts are but one example. Because different substances have different electrical properties, the conditions for release vary with each bound molecular species. In general, to get good separation, the methods of choice are either continuous ionic strength gradient elution or stepwise elution. (A gradient of pH alone is not used because it is difficult to set up a pH gradient without simultaneously increasing ionic strength.) For an anion exchanger, either pH and ionic strength are gradually increased or ionic strength alone is increased. The actual choice of the elution procedure is usually a result of trial and error and of considerations of stability. For example, for unstable materials, it is best to maintain fairly constant pH.

An ion exchanger is a solid that has chemically bound charged groups to which ions are electrostatically bound: it can exchange these ions for ions in aqueous solution. Ion exchangers can be used in column chromatography to separate molecules according to charge.; actually other features of the molecule are usually important so that the chromatographic behavior is sensitive to the charge density, charge distribution, and the size of the molecule.

The principle of ion-exchange chromatography is that charged molecules adsorb to ion exchangers reversibly so that molecules can be bound or eluted by changing the ionic environment. Separation on ion exchangers is usually accomplished in two stages: first, the substances to be separated are bound to the exchanger, using conditions that give stable and tight binding; then the column is eluted with buffers of different pH, ionic strength, or composition and the components of the buffer compete with the bound material for the binding sites.

An ion exchanger is usually a three-dimensional network or matrix that contains covalently linked charged groups. A typical group used in cation exchangers is the sulfonic group, SO_3^- . If an H^+ is bound to the group, the exchanger is said to be in the acid form; it can, for example, exchange on H^+ for one Na^+ or two H^+ for one Ca^{2+} . The sulfonic acid group is called a strongly acidic cation exchanger. Other commonly used groups are phenolic hydroxyl and carboxyl, both weakly acidic cation exchangers. If the charged group is positive - for example, a quaternary amino group--it is a strongly basic anion exchanger. The most common weakly basic anion exchangers are aromatic or aliphatic amino groups.

The matrix can be made of various material. Commonly used materials are dextran, cellulose, agarose and copolymers of styrene and vinylbenzene in which the divinylbenzene both cross-links the polystyrene strands and contains the charged groups. Table 1 gives the composition of many ion exchangers.

The total capacity of an ion exchanger measures its ability to take up exchangeable groups per milligram of dry weight. This number is supplied by the manufacturer and is important because, if the capacity is exceeded, ions will pass through the column without binding.

TABLE 1

Matrix	Exchanger	Functional Group	Tradename
Dextran	SC	Sulfopropyl	SP-Sephadex
	WC	Carboxymethyl	CM-Sephadex
	SA	Diethyl-(2-hydroxypropyl)-aminoethyl	QAE-Sephadex
	WA	Diethylaminoethyl	DEAE-Sephadex
Cellulose	C	Carboxymethyl	CM-Cellulose
	C	Phospho	P-cel
	A	Diethylaminoethyl	DEAE-cellulose
	A	Polyethylenimine	PEI-Cellulose
	A	Benzoylated-naphthoylated.deiethylaminoethyl	DEAE(BND)-cellulose
	A	p-Aminobenzyl	PAB-cellulose
Styrene-divinyl-benzene	SC	Sulfonic acid	AG 50
	SA		AG 1
	SC	Sulfonic acid +	AG 501
	+	Tetramethylammonium	
	SA		
Acrylic	WC	Carboxylic	Bio-Rex 70
Phenolic	SC	Sulfonic acid	Bio-Rex 40
Expoxyamine	WA	Tertiary amino	AG-3

The available capacity is the capacity under particular experimental conditions (i.e., pH, ionic strength). For example, the extent to which an ion exchanger is charged depends on the pH (the effect of pH is smaller with strong ion exchangers). Another factor is ionic strength because small ions near the charged groups compete with the sample molecule for these groups. This competition is quite effective if the sample is a macromolecule because the higher

diffusion coefficient of the small ion means a greater number of encounters. Clearly, as buffer concentration increases, competition becomes keener.

5 The porosity of the matrix is an important feature because the charged groups are both inside and outside the matrix and because the matrix also acts as a molecular sieve. Large molecules may be unable to penetrate the pores; so the capacity will decrease with increasing molecular dimensions. The porosity of the polystyrene-based resins is determined by the amount of cross-linking by the divinylbenzene (porosity decreases with increasing amounts of divinylbenzene). With the Dowex and AG series, the percentage of divinylbenzene is indicated
10 by a number after an X - hence, Dowex 50-X8 is 8% divinylbenzene.

Ion exchangers come in a variety of particle sizes, called mesh size. Finer mesh means an increased surface-to-volume ration and therefore increased capacity and decreased time for exchange to occur for a given volume of the exchanger. On the other hand, fine mesh means a
15 slow flow rate, which can increase diffusional spreading. The use of very fine particles, approximately 10 μm in diameter and high pressure to maintain an adequate flow is called *high-performance* or *high-pressure liquid chromatography* or simply HPLC.

Such a collection of exchangers having such different properties - charge, capacity,
20 porosity, mesh - makes the selection of the appropriate one for accomplishing a particular separation difficult. How to decide on the type of column material and the conditions for binding and elution is described in the following sections.

There are a number of choice to be made when employing ion exchange
25 chromatography as a technique. The first choice to be made is whether the exchanger is to be anionic or cationic. If the materials to be bound to the column have a single charge (i.e., either plus or minus), the choice is clear. However, many substances, carry both negative and positive charges and the net charge depends on the pH. In such cases, the primary factor is the stability of the substance at various pH values.

30 The choice between strong and weak exchangers is also based on the effect of pH on charge and stability. For example, if a weakly ionized substance that requires very low or high

pH for ionization is chromatographed. a strong ion exchanger is called for because it functions over the entire pH range. The pH at which the substance is stable must, of course, be matched to the narrow range of pH in which a particular weak exchanger is charged. Weak ion exchangers are also excellent for the separation of molecules with a high charge from those with a small charge, because the weakly charged ions usually fail to bind. Weak exchangers also show greater resolution of substances if charge differences are very small. If a macromolecule has a very strong charge, it may be also impossible to elute from a strong exchanger and a weak exchanger again may be preferable. In general, weak exchangers are more useful than strong exchangers.

The Sephadex and Bio-gel exchangers offer a particular advantage for macromolecules that are unstable in low ionic strength. Because the cross-links in these materials maintain the insolubility of the matrix even if the matrix is highly polar, the density of ionizable groups can be made several times greater than is possible with cellulose ion exchangers. The increased charge density means increased affinity so that adsorption can be carried out at higher ionic strengths.

Small molecules are best separated on matrices with small pore size (high degree of cross-linking) because the available capacity is large, whereas macromolecules need large pore size. However, except for the Sephadex type, most ion exchangers do not afford the opportunity for matching the porosity with the molecular weight.

The cellulose ion exchangers have proved to be the best for purifying large molecules such as proteins and polynucleotides. This is because the matrix is fibrous, and hence all functional groups are on the surface and available to even the largest molecules. In many cases however, beaded forms such as DEAE-Sephacel and DEAE-Biogel P are more useful because there is a better flow rate and the molecular sieving effect aids in separation.

Selecting a mesh size is always difficult. Small mesh size improves resolution but decreases flow rate, which increases zone spreading and decreases resolution. Hence, the appropriate mesh size is usually determined empirically.

Because buffers themselves consist of ions, they can also exchange, and the pH equilibrium can be affected. To avoid these problems, the *rule of buffers* is adopted: use *cationic buffers with anion exchangers* and *anionic buffers with cation exchangers*. Because ionic strength is a factor in binding, a buffer should be chosen that has a high buffering capacity so that its ionic strength need not be too high. Furthermore, for best resolution, it has been generally found that the ionic conditions used to apply the sample to the column (the so-called *starting conditions*) should be near those used for eluting the column.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

B) Purification of Phosphorothioate and Phosphorodithioate Oligonucleotides

Amino acid coupled oligonucleotides is purified using an anion exchange resin (Qiagen, Chatsworth, CA). The dried oligonucleotide is dissolved in 50 mM MOPS (pH 7.0) plus 15% ethanol and applied to an anion exchange column preequilibrated in the same buffer. The column will first be washed with 10 column volumes of 50 mM MOPS (pH 7.0) and 15% ethanol followed by 10 column volumes of 50mM MOPS (pH 7.0), 0.2 M NaCl and 15% ethanol. The oligonucleotide will be eluted with 50mM MOPS (pH 7.0), 2.0 M NaCl and 15% ethanol. The positive fractions are pooled and dialyzed against distilled water. After dialysis, the oligonucleotide concentration is determined at absorbance A_{254} . The concentration of the oligonucleotides is determined using the following formulas (Eckerstein, 1991): 1) micromolar extinction coefficient = $(8.8 \times nT) + (7.3 \times nC) + (11.7 \times nG) + (15.4 \times nA) \times 0.9$ and 2) the molecular weight = $(251 \times nA) + (245 \times nT) + (267 \times nG) + (230 \times nC) + (78 \times n-1) + (54 \times n) + (17 \times n-1) \div 2$. The oligonucleotide may then be lyophilized (Freeze dryer, VirTis, Gardiner, NY) and resuspended in sterile distilled water at a concentration of 10 mg/ml.

C) *Purification of Methylphosphonate Oligonucleotides*

Methylphosphonate oligonucleotides are purified on a DEAE column (Miller *et al.*, 1986). After de-protection with 30% ammonium hydroxide, the oligonucleotide is lyophilized, resuspended in 25% ethanol in water and passed through a DEAE-cellulose column. The column is washed with water and the oligonucleotide eluted with 0.15 M ammonium bicarbonate. The A₂₆₀ positive fraction is combined and the buffer is removed by lyophilization. The dried oligonucleotide is redissolved in sterile distilled H₂O at a concentration of 10 mg/ml.

4. **Assaying for Oligonucleotides of the Present Invention**

The Shine-Dalgarno region of prokaryotic organisms varies between about 3 and about 9 bases, forming a consensus sequence, for protein synthesis in such organisms. Of course, there may be variation in the Shine-Dalgarno sequence from one species to another. As such it will be necessary to identify oligonucleotides that will bind to the Shine-Dalgarno region of a given bacterial organism. It is well within the skill of the ordinary individual in the art, and will not involve undue experimentation, to produce a battery of oligonucleotides of 3, 4, 5, 6, 7, 8, 9 or more bases in length, in accordance with the present invention that will pair with the Shine-Dalgarno region of a given bacterial organism. The synthesis of such oligonucleotides has been discussed elsewhere in the specification.

In certain embodiments, the present invention concerns a method for identifying such additional oligonucleotides that will act as inhibitors of bacterial growth and infection. It is contemplated that this screening technique will prove useful in the screening of any oligonucleotide that is likely to compete with the Shine-Dalgarno region of prokaryotic microorganisms and that will serve the purpose of inhibiting the growth and attachment of such an organism.

Once the oligonucleotide (derivatized or underivatized), has been identified as capable of binding to the appropriate bacterial RNA, it will be necessary to screen it for an appropriate biological effect. Accordingly, the present section is directed to a method for determining the ability of a candidate oligonucleotide to inhibit the growth of a prokaryotic organism, in order to identify oligonucleotides useful in the present invention. The simplest and fastest method to

screen the biological effects of proposed oligonucleotides is by employing a growth assay. The method includes generally the steps of:

- (a) growing a microorganism in culture;
- (b) admixing a candidate oligonucleotide with the culture; and
- (c) determining the ability of the candidate oligonucleotide to inhibit the growth of the microorganism.

In an exemplary embodiment, *P. aeruginosa* is grown to stationary phase in M9 minimal media with glucose for 18 h at 37°C. The 18 h *P. aeruginosa* culture is diluted 1:10,000 in M9 minimal media with glucose containing varying amounts of oligonucleotide (0-50 µM) and then be incubated 24 h at 37°C. After a 24 h incubation, growth is determined by absorbance at A₆₀₀ using an ELISA plate reader (Titertek Multiskan, Flow Laboratories, McLean, VA). In each test, a control oligonucleotide may be used, in one such case the control oligonucleotide is a scrambled sequence of the SD oligonucleotide (nonsense oligonucleotide).

Microorganism growth impairment is expressed as a growth impairment (GI) index, similar to that described by Limper and Martin (Limper and Martin II, 1990), where 100% represents total inhibition of growth and 0% represents normal growth. The following formula is employed:

GI Index = [(A-B)/A] x 100% where

A = microorganism growth with no oligonucleotide as monitored by A₆₆₀ and
B = microorganism growth in the presence of candidate oligonucleotide as monitored by A₆₆₀.

Thus to identify a candidate oligonucleotide as being capable of inhibiting the growth of a microorganism, one would measure or determine the growth in the absence of the added candidate oligonucleotide. One would then compare this to the growth of a microorganism in the presence of the candidate oligonucleotide. A candidate oligonucleotide which inhibits the

growth relative to the growth of the organism in its absence is indicative of a candidate oligonucleotide with inhibitor capability useful in the present invention.

"Effective amounts" in certain circumstances are those amounts effective to reproducibly reduce the growth of the microorganism, in comparison to their normal levels. Oligonucleotides that achieve significant appropriate changes in growth will be used.

Significant decrease in growth, *e.g.*, as measured the growth assay mentioned above, of at least about 30%-40%, and most preferably, by decreases of at least about 50%, with higher values of course being possible.

In a particular embodiment, the present invention is concerned with a method of inhibiting the attachment of microorganism to normal human bronchial (NHB) cells. This method includes admixing NHB cells with for example *P. aeruginosa* organisms and monitoring attachment of the *P. aeruginosa* to the NHB cells. The candidate oligonucleotide may be added to a similar set of cells and the difference in attachment between the two sets of cells can be monitored. Useful candidate substance will decrease the attachment of *P. aeruginosa* organisms to the NHB cells. This is, of course, an important aspect of the invention in that it is believed that by inhibiting the attachment of microorganism to their target cells, one will be enabled to treat various aspects microbial infection by diminishing the amount of such infection. The use of such oligonucleotides to block the attachment of microorganisms will serve as a treatment for such infection. These oligonucleotides may thus be used in combination with conventional antibiotic/antimicrobial agents to treat multidrug resistant microbial infection. As such SD oligonucleotides are useful in conjunction with other antimicrobial therapies. Antimicrobial therapies are well known to those of skill in the art and the Skilled artisan is referred to "Remington's Pharmaceutical Sciences" for exemplary antimicrobial agents that may be used in conjunction with the methods and therapies described herein.

5. Delivery of the Oligonucleotide

Cystic fibrosis is the most common lethal inheritable disease in Caucasians (Collins, 1992) and *P. aeruginosa* is a major opportunistic infection associated with this disease. The

initial infection is believed to occur with the nonmucoid phenotype in the upper respiratory tract in cystic fibrosis patients (Govan, 1988). Once the infection is established, it has been speculated that the nonmucoid strain changes to the mucoid strain as a way to survive the "hostile" environment in the lower airways of the lung (Gilligan, 1991). The mucoid phenotype is characterized by an alginate polysaccharide capsule containing proteins and nucleic acids. This capsule protects the colony by forming a barrier to alveolar macrophage, neutrophils and antibodies. Once a mucoid infection is established, it is rarely eradicated (Editorial, 1986). As a result, *P. aeruginosa* infection leads to a slow deterioration of pulmonary function and usually ends in patient death (Kerem *et al.*, 1990; Winnie and Cowan, 1991).

The present invention provides compositions and methods of treatment of pulmonary diseases such as cystic fibrosis and TB. Even when there is a plethora of treatment regimens for a disease the mode of administration of a drug can affect its bioavailability and pharmacokinetics profile as well as patient compliance. Patient compliance is best when the mode of administration is convenient and involves the least amount of discomfort. As such, oral administration is often the most preferred modes.

Despite its obvious advantages, however, oral administration is unworkable in many cases, including the hindrance of metabolism during transit from the gastrointestinal tract to the general circulation. Thus bioavailability of orally transmitted compositions is low.

Pulmonary delivery offers several potential advantages, particularly in the case of administration of compositions intended to treat conditions affecting the lungs because such compositions often have difficulty reaching the lungs by any route of administration. The treatment of cystic fibrosis has always been a challenge since antibiotics delivered by conventional modes of administration do not easily reach the lungs and thus can not stop the spread of infection.

Pulmonary drug delivery methods include mechanical means such as aerosols and inhalers. Such delivery methods often employ compositions encapsulated in liposomes. There is a wide body of knowledge that teaches the use of liposomal compositions to delivery pharmaceutically therapeutic compositions to the alveolar surface. The skilled artisan is

referred to PCT patent publication numbers WO/90/07469; WO 96/32116; WO 96/27393; WO 93/12240 and WO 96/22765 (incorporated herein by reference) for exhaustive treatise on the delivery of such compositions to the lung.

5 The present invention employs liposomal delivery of the therapeutic compositions to the lung surface for the treatment of diseased conditions such as cystic fibrosis and TB. By way of example, the preparation of liposomes is described herein below, however it is understood that the liposomal compositions described herein are only exemplary and the skilled artisan may employ any liposomal composition effective for the purposes of drug delivery in order to
10 deliver the therapeutic compositions of the present invention to their site of action/effect.

 In particular embodiments it is envisioned that the therapeutic compositions of the present invention will be targeted to intracellular pathogens by employing liposomal entrapment of the competitive oligonucleotide suspended in a sterile aqueous medium. In preferred
15 methods of the invention, these liposome compositions are delivered to the lower respiratory tract (including the lungs) of the subject undergoing treatment *via* an aerosol administered through the mouth and/or nose. Such methods may employ direct intratracheal instillation with mechanical ventilation, and/or the use of jet or ultrasonic nebulization to create an aerosol which is introduced to the lower respiratory tract *via* the mouth and/or nose of the subject. The
20 entrapment of the competitive oligonucleotide in a liposome adapted to be engulfed by AMs can thus be used to facilitate delivery of the oligonucleotide to spaces within the AMs for treatment of infections by bacterial organisms.

Liposomal Formulations

25 In a preferred embodiment of the invention, the oligonucleotides may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the
30 formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are cationic lipid-nucleic acid complexes, such as lipofectamine-nucleic acid complexes.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated oligonucleotide (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression vectors have been successfully employed in transfer and expression of a polynucleotide *in vitro* and *in vivo*, then they are applicable for the present invention.

"Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers. Phospholipids are used for preparing the liposomes according to the present invention and can carry a net positive charge, a net negative charge or are neutral. Dicetyl phosphate can be employed to confer a negative charge on the liposomes, and stearylamine can be used to confer a positive charge on the liposomes.

Lipids suitable for use according to the present invention can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma Chemical Co., dicetyl phosphate ("DCP") is obtained from K & K Laboratories (Plainview, NY); cholesterol ("Chol") is obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, Ala.). Stock solutions of lipids in chloroform, chloroform/methanol or *t*-butanol can be stored at about -20°C. Preferably, chloroform is used as the only solvent since it is more readily evaporated than methanol.

Phospholipids from natural sources, such as egg or soybean phosphatidylcholine, brain phosphatidic acid, brain or plant phosphatidylinositol, heart cardiolipin and plant or bacterial phosphatidylethanolamine are preferably not used as the primary phosphatide, *i.e.*, constituting 50% or more of the total phosphatide composition, because of the instability and leakiness of the resulting liposomes.

Liposomes used according to the present invention can be made by different methods. The size of the liposomes varies depending on the method of synthesis. A liposome suspended in an aqueous solution is generally in the shape of a spherical vesicle, having one or more concentric layers of lipid bilayer molecules. Each layer consists of a parallel array of molecules represented by the formula XY, wherein X is a hydrophilic moiety and Y is a hydrophobic moiety. In aqueous suspension, the concentric layers are arranged such that the hydrophilic moieties tend to remain in contact with an aqueous phase and the hydrophobic regions tend to self-associate. For example, when aqueous phases are present both within and without the liposome, the lipid molecules will form a bilayer, known as a lamella, of the arrangement XY-YX.

Liposomes within the scope of the present invention can be prepared in accordance with known laboratory techniques. In one preferred embodiment, liposomes are prepared by mixing liposomal lipids, in a solvent in a container, *e.g.*, a glass, pear-shaped flask. The container should have a volume ten-times greater than the volume of the expected suspension of liposomes. Using a rotary evaporator, the solvent is removed at approximately 40°C under negative pressure. The solvent normally is removed within about 5 min to 2 hours, depending on the desired volume of the liposomes. The composition can be dried further in a desiccator under vacuum. The dried lipids generally are discarded after about 1 week because of a tendency to deteriorate with time.

Dried lipids can be hydrated at approximately 25-50 mM phospholipid in sterile, pyrogen-free water by shaking until all the lipid film is resuspended. The aqueous liposomes can be then separated into aliquots, each placed in a vial, lyophilized and sealed under vacuum.

In the alternative, liposomes can be prepared in accordance with other known laboratory procedures: the method of Bangham *et al.* (1965), the contents of which are incorporated herein by reference; the method of Gregoriadis, as described in *DRUG CARRIERS IN BIOLOGY AND MEDICINE*, G. Gregoriadis ed. (1979) pp. 287-341, the contents of which are incorporated herein by reference; the method of Uster and Deamer (1983), the contents of which are incorporated by reference; and the reverse-phase evaporation method as described by Szoka and

Papahadjopoulos (1978). The aforementioned methods differ in their respective abilities to entrap aqueous material and their respective aqueous space-to-lipid ratios.

5 The dried lipids or lyophilized liposomes prepared as described above may be reconstituted in a solution of nucleic acid and diluted to an appropriate concentration with an suitable solvent, *e.g.*, DPBS. The mixture is then vigorously shaken in a vortex mixer. Unencapsulated nucleic acid is removed by centrifugation at $29,000 \times g$ and the liposomal pellets washed. The washed liposomes are resuspended at an appropriate total phospholipid concentration, *e.g.*, about 50-200 mM. The amount of nucleic acid encapsulated can be
10 determined in accordance with standard methods. After determination of the amount of nucleic acid encapsulated in the liposome preparation, the liposomes may be diluted to appropriate concentration and stored at 4°C until use.

15 In a preferred embodiment, the lipid dioleoylphosphatidylchoine is employed. Nuclease-resistant oligonucleotides were mixed with lipids in the presence of excess t-butanol. The mixture was vortexed before being frozen in an acetone/dry ice bath. The frozen mixture was lyophilized and hydrated with Hepes-buffered saline (1 mM Hepes, 10 mM NaCl, pH 7.5) overnight, and then the liposomes were sonicated in a bath type sonicator for 10 to 15 min. The size of the liposomal-oligonucleotides typically ranged between 200-300 nm in diameter as
20 determined by the submicron particle sizer autodilute model 370 (Nicomp, Santa Barbara, CA).

Regardless of the liposome formulation, liposomes will be modified to enhance delivery to the target host cells. For example, mycobacterial species typically use alveolar macrophages as a host cell within which to replicate and thrive. In these circumstances, the liposome
25 containing the oligonucleotide will be modified so that the alveolar macrophage and secondarily to the phagolysosome containing the mycobacteria. As an example, the inventors coat liposome with immunoglobulins which will facilitate targeting to the alveolar macrophages via the Fc receptor. As liposomes coated with immunoglobulins are attached to the macrophage via the Fc receptor, the liposomes will be rapidly internalized and directed to the phagolysosome (Buchi et al., Cell Structure and Function 18:399-407, 1993). Alternatively, liposomes can be coated
30 with mannose or mannose complexes to facilitate recognition and internalization by alveolar

macrophages via the mannose receptor. Similarly, the mannose receptor will deliver the liposome to the phagolysosome of the macrophage (Prigozy et al., Immunity 6:187-197, 1997).

6. Combined Therapy Protocols.

5 Multidrug resistant strains of bacteria represents a significant challenge in the treatments of infection. One goal of the current anti-microbial research is to find ways to improve the efficacy of the currently available therapeutic agents. In the context of the present invention, it is contemplated that the competitive oligonucleotides could be used in conjunction with more traditional antimicrobial intervention.

10 To kill the microbial cells, such as *P. aeruginosa*, *M. avium* and the like, using the methods and compositions of the present invention, one would generally contact a "target" area of microbial infection, for example in the lung for alveolar macrophages with an oligonucleotide composition of the present invention and at least one antimicrobial agent.

15 These compositions would be provided in a combined amount effective to kill or inhibit the infective potential of that microorganism. This process may involve contacting the area with the SD composition and the antimicrobial agent(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same

20 time, wherein one composition includes the SD composition and the other includes the antimicrobial agent.

Alternatively, the oligonucleotide treatment may precede or follow the antimicrobial agent treatment by intervals ranging from minutes to weeks. In embodiments where the

25 antimicrobial factor and oligonucleotide are applied separately to the microorganism, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the antimicrobial agent and oligonucleotide would still be able to exert an advantageously combined effect on the microorganism. In such instances, it is contemplated that one would contact the microorganism with both agents within about 6 hours to one week of

30 each other and, more preferably, within about 24-72 hours of each other, with a delay time of only about 48 hours being most preferred. In some situations, it may be desirable to extend the

time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either the oligonucleotide or the antimicrobial agent will be desired. Various combinations may be employed, where the SD oligonucleotide is "A" and the antimicrobial agent is "B":

A/B/A	B/A/B	B/B/A	A/A/B	A/B/B
B/A/A	B/B/B/A	B/B/A/B	A/A/B/B	A/B/A/B
A/B/B/A	B/B/A/A	B/A/B/A	B/A/A/B	A/A/A/B
B/A/A/A	A/B/A/A	A/A/B/A	A/B/B/B	B/A/B/B

To achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the microorganism.

Antimicrobial agents are defined herein as any chemical compound or treatment method that is effective at reducing bacterial infection in a host or that is capable of reducing or inhibiting the growth of a bacterial organism in culture. Such agents are well known to those of skill in the art and the skilled artisan is referred to Table 44.1 and page 1018-1164 in Goodman and Gilman's "Pharmacological Basis of Therapeutics" (Gilman *et al.*, 1990, incorporated herein by reference). In particular embodiments, the antimicrobial agent may be a sulfonamide, a quinolone, a penicillin, a cephalosporin, a beta-lactam antibiotic, an aminoglycoside, or a tetracycline.

In treating a microbial infection according to the invention, one would contact the infected organism with an antimicrobial agent in addition to the oligonucleotide composition. This may be achieved by contacting the infected organism or the infecting micro-organism with the anti-microbial agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising an anti-microbial agent such as penicillin, streptomycin, ampicillin, ceftriaxone, gentamicin, rifampin, amoxicillin, erythromycin, aminoglycosides, cephalosporins, and tetracyclines. These and other antimicrobial agents are well known to those of skill in the art, and the skilled artisan is referred to text such as

Physicians Desk Reference and Remington's Pharmaceutical Sciences" 15th Edition for specific doses and indications. The antimicrobial agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with an oligonucleotide composition of the present invention.

5

Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

10

The inventor proposes that the regional delivery of SD directed oligonucleotides to patients with bacterial infection will be a very efficient method for delivering a therapeutically effective composition to counteract the clinical disease. Similarly, the antimicrobial agent may be directed to a particular, affected region of the subject's body. Alternatively, systemic delivery of the oligonucleotide or the anti-microbial agent may be appropriate in certain circumstances, for example, where extensive infection has occurred.

15

7. **Pharmaceutical Compositions and Routes of Administration**

Where clinical application of liposomes containing oligonucleotides is undertaken, it will be necessary to prepare the liposome complex as a pharmaceutical composition appropriate for the intended application. Generally, this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate buffers to render the complex stable and allow for uptake by target cells.

20

25

Aqueous compositions of the present invention comprise an effective amount of the antisense oligonucleotide encapsulated in a liposome as discussed above, further dispersed in pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate.

30

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of therapeutic compositions can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like.

Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch,

magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, capsules, sustained release formulations or powders.

5 The therapeutic compositions of the present invention may include classic pharmaceutical preparations. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration will be by orthotopic, intradermal subcutaneous, intramuscular, intraperitoneal or intravenous
10 injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. For treatment of conditions of the lungs, the preferred route is aerosol delivery to the lung. Volume of the aerosol is between about 0.01 ml and 0.5 ml. Similarly, a preferred method for treatment of colon-associated disease would be via enema. Volume of the enema is between about 1 ml
15 and 100 ml. Pulmonary administration of pharmaceutically active substances via aerosolization is a well known technique to those of skill in the art and the skilled artisan is referred to PCT publications WO 90/07469; WO 96/32116; WO 96/27939 WO 93/12240 for further details on the preparation of such compositions.

20 An effective amount of the therapeutic composition is determined based on the intended goal. The term "unit dose" or "dosage" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to
25 number of treatments and unit dose, depends on the protection desired.

Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting dose include physical and clinical state of the patient, the route of administration, the intended goal of treatment
30 (alleviation of symptoms *versus* cure) and the potency, stability and toxicity of the particular therapeutic substance. For the instant application, it is envisioned that the amount of

therapeutic composition comprising a unit dose will range from about 5-30 mg of oligonucleotide.

8. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

METHODS

Synthesis of Oligonucleotides

The oligonucleotides will be synthesized on a Milligen/Bioscience cyclone DNA synthesizer (Millipore, Marlborough, MA) using the β -cyanoethyl phosphoramidite method for DNA synthesis (Eckstein, 1991). All reagents used for DNA synthesis will be obtained from Glen Research (Sterling, VA). For phosphorothioate oligonucleotides, Beaucage reagent will be used during the oxidation step. For methylphosphonate oligonucleotides, methylphosphonate monomers will be used as a precursor during the synthesis.

P. aeruginosa Culture Conditions

P. aeruginosa (nonmucoid strain, PAO 1 ATCC 15692, American Type Culture Collection, Rockville, MD) will be grown with aeration in M9 minimal media plus glucose at 37°C (Maniatis *et al.*, 1989). *P. aeruginosa* (mucoid strain, ATCC 17933) will be maintained in the mucoid state by growing the organisms in M9 media adjusted to 0.3 M NaCl. *P. aeruginosa* organisms will be quantified by measuring the absorbance at A_{600} . The optical density of 1.0 at 600 nm corresponds to 6.0×10^8 bacteria/ml (Simpson *et al.*, 1992; Doudorf and Palleroni). Screening for the mucoid and nonmucoid phenotypes will be done using standard morphological and biochemical tests (Doudorf and Palleroni).

Human Epithelial Cell Culture

Human A549 epithelial cells (ATCC #CCL 185) will be cultured as monolayers in standard T75 culture flasks in RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 10% heat-activated fetal bovine serum (Hyclone, Laboratories, Logan, UT), penicillin (10 µg/ml), gentamycin (4 µg/ml), and amphotericin (0.5 µg/ml). Cells will be transferred to 96 well tissue culture plates for bacterial adherence assays, 24 well plates for ³⁵S-labeling assays, and 6 well plates for cell growth inhibition assays.

Normal human bronchial (NHB) epithelial cell cultures will be supplied by Dr. Debra J. Romberger. Cells will be grown on Vitrogen 100 (Collagen Corporation, Palo Alto, CA)-coated tissue culture plastic and plated with a 1:1 mixture of LHC-9 and RPMI 1640 (Biofluids, Inc., Rockville, MD) containing Fungizone (2.5 ml/L) and PenStrep (2.5 ml/L) as described previously (Lechner and LaVeck, 1985). Cells will be transferred to 96 well plates for bacterial adherence assays, 24 well plate for ³⁵S-labeling assays, and 6 well plates for cell growth inhibition assays.

Effect of Oligonucleotides in *P. aeruginosa* Growth

P. aeruginosa will be grown to stationary phase in M9 minimal media with glucose for 18 h at 37°C. The 18 h *P. aeruginosa* culture will be diluted 1:10,000 in M9 minimal media with glucose containing varying amounts of oligonucleotide (0-50 µM) and then be incubated 24 h at 37°C. After a 24 h incubation, growth will be determined by absorbance at A₆₀₀ using an ELISA plate reader (Titertek Multiskan, Flow Laboratories, McLean, VA). In each study, the control oligonucleotide will be a scrambled sequence of the SD oligonucleotide with the following sequence: 5'-(amino acid)-AGG TGA CCC TCG TGA GAG AC 3' (SEQ ID NO:7). This oligonucleotide with the scrambled sequence will be referred to as the nonsense oligonucleotide. oligonucleotides with all 20 naturally occurring amino acids will be coupled to the 5' end of the oligonucleotide and evaluated by growth assays.

P. aeruginosa growth impairment will be expressed as a growth impairment index, similar to that described by Limper and Martin (Limper and Martin II, 1990). The epithelial (A549 and HNB) cells will be plated at a density of 30,000 cells/well in a 6-well plate. After attachment of the epithelial cells to the tissue culture plate, 50 µM of SD and nonsense

oligonucleotide will be added to the wells. Wells without added oligonucleotide will serve as a negative control in addition to the nonsense oligonucleotide. After 72 h, the medium will be removed, and the monolayers will be washed thoroughly $\times 3$ with 2 ml of Hanks' buffered saline solution. The epithelial cells will be removed with Trypsin Versene (Biofluids, Rockville, MD) and enumerated by Coulter counter analysis (Model ZM, Coulter Corp, Hialeah, FL). Epithelial cell growth impairment will be expressed as a growth impairment (GI) index, where 100% represents total inhibition of growth and 0% represents normal growth. The following formula will be employed: $GI\ Index = [(A-B)/A] \times 100\%$, where A = epithelial cell count in wells with no oligonucleotide and B = epithelial cell count in wells containing oligonucleotide.

Fluorescent-Oligonucleotide Transport Assay

Fluorescein isothiocyanate (FITC)-labeled oligonucleotides can be synthesized using columns with a solid support containing a 3'-FITC (Glen Research, Sterling, VA). In order to test which amino acid will permit the most efficient transport, 3'-FITC-5'-amino acid oligonucleotides will be synthesized with a different amino acid on each batch. An 18 h culture of *P. aeruginosa* in M9 minimal media with glucose will be diluted 1:10 in fresh media containing FITC-labeled oligonucleotide. The *P. aeruginosa* organisms will be incubated 18 h. The next day the *P. aeruginosa* organisms will be spin washed $\times 3$ (12,000 g \times 5 min) in M9 minimal media with the aid of a micro centrifuge. A 100 μ l aliquot of each sample will be added to a 96 well plate. The fluorescence will be measured on a CytoFluor fluorescent plate reader (Millipore, Marlborough, MA) set at an excitation of 485 nm and an emission of 530 nm. An increase in fluorescence will represent an increase in transport of the fluorescently-labeled oligonucleotide.

Confocal Laser Scanning Microscopy

To verify the transport of the oligonucleotides into *P. aeruginosa*, confocal scanning microscopy will be used (Yosida *et al.*, 1992; Shotton, 1989; Bakewell *et al.*, 1991). Briefly, samples of *P. aeruginosa* previously incubated with FITC-labeled oligonucleotide as above, will be mounted and air dried on a microscope slide for examination under a confocal microscope. The confocal microscope system and technical support, which is available in the Pulmonary core facility at Indiana University is equipped with an InSight Plus real-time

scanning module and cooled argon-ion and krypton laser systems which includes an argon-krypton multi-color filter wheel (Meridian Instruments, Okemos, MI). Images seen through a Zeiss Axioplan microscope (Carl Zeiss Inc., Thornwood, NY) equipped with high numerical aperture objectives ideal for confocal microscopy, are captured *via* a cooled CCD video detector system and projected on a high resolution monitor. The fluorescent images can be visualized and quantified with the aid of image processing and analysis software. A Z-drive accessory component enables optical sectioning under computer control with a minimum vertical step of 0.1 μm . These sections can be used to reveal the locale of the incorporated fluorescent label, provide accurate determination of length, width, and depth of specific cells, and create a 3-D image of the specimen, providing confocal microscopy a distinct advantage over standard epifluorescence microscopy.

Protein Synthesis Inhibition Assay as Determined by ^{35}S -Labeling of Proteins

A stationary phase culture of *P. aeruginosa* grown in M9 minimal media with glucose will be diluted 1:1000 in 0.5 ml of fresh media with and without oligonucleotides. After the preincubation with Shine-Dalgarno (SD), nonsense and no oligonucleotides, a pulse label using 25 μCi of Trans ^{35}S -label[®] (ICN, Costa Mesa, CA) (Trans ^{35}S -label[®] contains ^{35}S -Met and ^{35}S -Cys) will be incubated with *P. aeruginosa* organisms for 18 h. The following day, the *P. aeruginosa* organisms will be pelleted and the supernatant saved for a trichloroacetic acid (TCA) precipitation assay to determine the amount of radioactivity associated with excreted protein. For the TCA precipitation assay, 50 μl of supernatant will be added to 0.5 ml of 0.1 mg/ml BSA containing 0.02% NaN_3 in a siliconized 1.5 ml eppendorf tube. After addition of ice cold 20% TCA 0.5 ml, the mixture will be vortexed and incubated for 30 min on ice. The precipitate will be filtered through glass fiber filters using a vacuum filter apparatus. The filters will be washed $\times 2$ with 5.0 ml ice cold 10% TCA, $\times 2$ with 100% ethanol and air dried. The filters will be counted in a β scintillation counter (LS 3801, Beckman Instruments, Irvine, CA).

Identical pulse labeling using Trans ^{35}S -label[®] will be performed with A549 cells and normal human bronchial (HNB) epithelial cells in the presence and absence of oligonucleotides. Following the pulse labeling, the A549 cells and HNB epithelial cells will be washed $\times 2$ with fresh media and lysed with 2% SDS solution. The lysed cells will be boiled for five min to solubilize the labeled proteins, and the cell lysates will be TCA precipitated as before.

Inhibition of protein synthesis, as assessed by a decrease in radioactive label in the precipitated proteins, will be compared between *P. aeruginosa* and the eukaryotic cells to ensure specificity of the oligonucleotides.

5 **Assessment of Oligonucleotide Transport as a Function of the Coupled Amino Acid**

In order to determine which amino acid will best facilitate transport, 3'-FITC-labeled oligonucleotides will be synthesized with the following 5'-amino acids: arginine, asparagine, glutamine, glycine, valine, isoleucine, leucine, methionine, proline, tryptophan and valine. The transport of each oligonucleotide with differing amino acids will be determined using the fluorescent transport assay. The transport will be determined in *P. aeruginosa* (strains PAO1 nonmucoid, FRD1 mucoid), A549 and INB epithelial cells. The cells will be incubated for time periods from 0 to 24 h with 50 μ M 3'-FITC, 5'-amino acid oligonucleotide. Cells will be sampled and washed at 1, 2, 4, 8, 12, and 24 h time points. The fluorescence will be quantified on a CytoFluor fluorescent plate reader. Controls will consist of the following: 1) cells with no added oligonucleotide 2) 3'-FITC-labeled oligonucleotides with no coupled amino acid and 3) 50 mM of free amino acid plus 50 μ M of the oligonucleotide modified with the same amino acid. These controls will be used to assess the background fluorescence and the effect of the 5'-amino acid on the transport mechanism. Sampling the cells at different time points as stated above during the incubation will provide kinetic data. Using fluorescent specific activity (fluorescent units per μ g of oligonucleotide) the rate of oligonucleotide transport will be quantified as mass of oligonucleotide/time $\times 10^6$ organisms.

Inhibition of Cellular Protein Synthesis by Amino Acid Modified Oligonucleotide

Each oligonucleotide with a specific amino acid coupled to the 5'-end (50 μ M) will be incubated with *P. aeruginosa* (mucoid and nonmucoid), A549 and NHB epithelial cells in the presence of 25 μ Ci Tran³⁵S-label[®]. To determine specificity, the protein assay will be conducted on both *P. aeruginosa* and the epithelial cells. *P. aeruginosa* organisms (2.5×10^5) in 0.5 ml of M9 media or lung epithelial cells (A549 or NHB) will be preincubated with and without oligonucleotides (50 μ M) for 4 h. After the preincubation, the cells will be pulsed labeled with 25 μ Ci of Trans ³⁵S-label[®] for 1 to 24 h for *P. aeruginosa* and eukaryotic cells. Following the pulse labeling, inhibition of protein synthesis in *P. aeruginosa*, A549 and NHB epithelial cells will be determined as outlined in General Methods. In each case, a nonsense

oligonucleotide with the same 5'-amino acid will be used as a negative control. A comparison of the percent of protein inhibition between *P. aeruginosa* and eukaryotic cells will give an indication of the specificity for the oligonucleotide.

EXAMPLE 2

PREPARATION AND TESTING OF COMPETITIVE OLIGONUCLEOTIDE

Using the recently published sequence for the 16S rDNA sequence. (van der Giessen *et al.*, 1994)) a competitive synthetic oligonucleotide containing the Shine-Dalgarno sequence was constructed on a cyclone plus DNA synthesizer (Biosearch) using standard phosphoramidite chemistry (Gart, 1986). The sequence contains the specific flanking regions encoded by the 16S rDNA for *M. avium* (SEQ ID NO:9).

The oligonucleotide was lyophilized and then resuspended in 10 mM Tris (pH 7.5), 1 mM EDTA (TE buffer) and quantified by measuring the absorbance at 260 nm. The oligonucleotide, at 50 μ M, was incubated with *M. avium* for 24 h and in aliquot assayed by a standard radiometric (BACTEC) (Reddy *et al.*, 1994).

In particular, in this method, mycobacterial metabolism is measured by the $^{14}\text{CO}_2$ released during the decarboxylation of ^{14}C -labeled fatty acid present in the medium. The amount of radioactivity released in each vial is determined quantitatively on a scale of 0-999 and is expressed as the growth index (GI) with a GI > 10 indicating metabolic growth. Briefly, diluted tissue homogenates or cell lysates (0.5 ml) are inoculated into BACTEC 12B vials (Becton Dickinson, San Jose, CA) containing 7H12 Middlebrook broth and an antimicrobial PANTA supplement used to prevent growth of any contaminating organisms. Each vial is filled with CO_2 and incubated at 37°C on the BACTEC instrument. The amount of radioactivity released into the head space is analyzed by the BACTEC instrument every 24 hours.

The results, shown in FIG. 1A, evidence the efficacy of the inventive method in inhibiting *M. avium* growth. Although the present invention is not limited by any theory, it is assumed that the mechanism of action is bacteriostatic, like many antibiotics, that *M. avium* growth might recover (oligo curve is increasing slightly at day 7 in this particular assay), and that multiple doses of oligonucleotide may be necessary.

In yet another exemplary study the inventors employed the oligonucleotides of the present invention to specifically inhibit the growth of *P. aeruginosa*. A 3'-FITC-labeled synthetic oligonucleotide was synthesized to demonstrate that alanine facilitates transport of the oligonucleotide into *P. aeruginosa* organisms. A 3'-FITC-labeled oligonucleotide (50µm) with and without a 5'-alanine residue was incubated overnight with *P. aeruginosa* and the transport of the oligonucleotide was measured using fluorimetry as described above. The increase in fluorescence as measured by a CytoFluor (Millipore, Marlborough, MA, FIG. 3) suggests that the 5'-alanine facilitates oligonucleotide transport into *P. aeruginosa* organisms. Transport of the FITC-labeled oligonucleotide may further be verified by confocal microscopy as described in Example 1.

EXAMPLE 3

BIOTINYLATION TESTING

To study the effects of biotinylation on the oligonucleotide was biotinylated at the 5' end of the oligonucleotide using biotin phosphoramidite precursor (Glen Research, Sterling, VA). In a first study, both the biotinylated and non-biotinylated oligonucleotides were FITC labeled, incubated with *M. avium* and uptake was quantified by a fluorescent plate reader (CytoFluor). In a second study, oligonucleotides (0-50 µM) with and without biotinylation were incubated with *M. avium* and an aliquot assayed by BACTEC as described in Example 2. The results are shown in FIG. 2A and FIG. 2B, respectively, and demonstrate that biotinylation of an oligonucleotide increases uptake by *M. avium* (FIG. 2A) and leads to a greater mycobacterial effect (FIG. 2B) as compared to the non-biotinylated form.

A nonsense oligonucleotide was also constructed and used in an analogous incubation procedure, and the biologic effect compared to the SD oligonucleotide. The results, shown in FIG. 1B, demonstrate that the effect of the SD oligonucleotide appears to be specific as a significant difference exists at each of the time points between the SD and nonsense oligonucleotides.

EXAMPLE 4

INHIBITION OF *P. AERUGINOSA* GROWTH BY THE SYNTHETIC
PHOSPHOROTHIOATE OLIGONUCLEOTIDE COUPLED TO A 5'-ALANINE

To demonstrate the effect on growth of the 5'-alanine coupled to the oligonucleotide, *P. aeruginosa* was cultured in minimal media with a phosphorothioate oligonucleotide with a 5'-alanine at various concentrations. *P. aeruginosa* was grown overnight in M9 minimal media with glucose. The overnight culture was diluted 1 to 10,000 in M9 minimal media with glucose containing varying concentrations of 5'-alanine phosphorothioate SD oligonucleotide targeted to *P. aeruginosa* 16S rRNA 3'-end. As a control, the sequence was scrambled and incubated in the same manner as the SD oligonucleotide. After an overnight incubation, growth was measured at A₆₂₀. All samples were done in triplicate.

The results in FIG. 5 indicate that the SD oligonucleotide (●) inhibits growth of *P. aeruginosa* organisms in a concentration-dependent manner while the control nonsense oligonucleotide (○) had no discernible effect on growth. This figure serves to demonstrate that the SD oligonucleotides of the present invention are effective at inhibiting the growth of microorganisms. It is anticipated that the several modifications as proposed in the current specification will offer the opportunity to increase transport, further increase specificity, and increase the antibacterial effect of the oligonucleotides on *Pseudomonas*.

To demonstrate the effect of alanine on the oligonucleotide's antimicrobial properties, *P. aeruginosa* was incubated in the presence of oligonucleotides with and without an alanine coupled to the 5'-end (FIG. 4). *P. aeruginosa* was grown overnight in M9 minimal media with glucose. The overnight culture was diluted 1 to 10,000 in M9 minimal media with glucose. The diluted *P. aeruginosa* organisms were incubated with: 1) 50 μM of SD oligonucleotide with a 5'-alanine, 2) 50 μM of SD oligonucleotide without a 5'-alanine, and 3) no oligonucleotides. Growth was measured by absorbance at A₆₂₀. The results show that coupling of 5'-alanine to the SD oligonucleotide further reduces growth of *P. aeruginosa* supporting the role of alanine in oligonucleotide transport.

EXAMPLE 5

REDUCTION OF *P. AERUGINOSA* TO EPITHELIAL CELLS BY SHINE-DALGARNO OLIGONUCLEOTIDES

To demonstrate that *Pseudomonas* attachment to epithelial cells can be quantified, ⁵¹Cr-labeled *P. aeruginosa* organisms were incubated with lung epithelial cells from 0 to 6 h. B. To demonstrate the effect of the SD oligonucleotide on the attachment to lung epithelial cells, ⁵¹Cr-labeled *P. aeruginosa* was incubated with lung epithelial cells in the presence and absence of SD oligonucleotide with a coupled 5'-alanine. As a further control, an oligonucleotide with no 5'-alanine was used in the assay (FIG. 6A and FIG. 6B). The data indicate that *Pseudomonas* attachment can easily be quantified and increases as a function of time with an apparent plateau between 4 and 6 hours (FIG. 6A). The SD oligonucleotide with or without 5'-alanine significantly reduced attachment under conditions that promote attachment. It is clear that modification of the oligonucleotide with a 5'-alanine significantly enhances the inhibitory effect of the SD oligonucleotides on *P. aeruginosa* attachment to epithelial cells.

EXAMPLE 6

PREPARATION AND DELIVERY OF LIPOSOMES

Anionic, multilamellar liposomes are prepared by aqueous reconstitution of phospholipids (dipalmitoylphosphatidylcholine, 63 μ mole, dicetyl phosphate 18 μ mole; cholesterol, 9 μ mole) in the presence of 2 ml of 0.3 mg/ml biotinylated oligonucleotide conjugated to fluorescein isothiocyanate followed by repetitive thermal cycling from -40 to +60°C. This preparation of anionic liposomes was chosen because it was nontoxic and rapidly phagocytosed by AMs. Cationic liposomes are also used as the incorporation of DNA is higher into cationic liposomes than anionic liposomes. That cationic liposomes are prepared using L- α -phosphatidylcholine (63 μ moles), stearylamine (18 μ moles) and cholesterol (9 μ moles) prepared under nitrogen in rotating glass vials. Either liposome suspension is centrifuged \times 5 (13,000 \times g 5 min); the supernatant is discarded and the pellet is resuspended in 5 ml Ca²⁺-free normal saline with each cycle of centrifugation. After being suspended in a final volume of 2 ml, samples are mounted on standard microscope slides and observed by differential interference contrast and epifluorescence microscopy using a Zeiss Axioplan fitted with a 35 mm camera. To prepare liposomes of defined size (2, 5, or 6 μ m), the multilamellar liposome

suspension is extruded through a polycarbonate membrane of appropriate pore size (Costar, Cambridge, MA) in a membrane extruder (Lipex Biomembranes, Vancouver, BC). Liposome number and size are determined by Coulter Counter and Channelyzer analysis.

Two methods are used for airway delivery of liposomes to the lower respiratory tract of rodents (mice and rats): 1) a method of direct intratracheal (I.T.) instillation using mechanical ventilation, and 2) use of ultrasonic nebulization to promote aerosolization in a modular 24 port "nose-only" device.

For mechanical ventilation, pathogen-free 8-10 week old mice are anesthetized with 3 mg ketamine (IM), tracheotomized using a 20 gauge angiocath, and ventilated using a small animal ventilator (Analytical Specialties, Inc., St. Louis, MO) (Harris *et al.*, 1989; Anderson *et al.*, 1992). The ventilator is time-cycled with pressure limits set at 4 cm H₂O and positive end-expiratory pressure set at 0 cm H₂O. Tidal volumes are typically 0.2-0.3 ml with a rate of 140-160/min resulting in minute ventilation of 30-40 ml. Peak inspiratory pressures are between 3-4 cm H₂O. The ventilator is designed to ventilate two animals simultaneously. To date, results from I.T. inoculated FITC-TB/*M. avium* have been most reproducible with ventilated animals compared to I.T. administration in briefly anesthetized (but not mechanically ventilated) animals. Mechanical ventilation insures adequate airway delivery of the labeled TB/*M. avium* organisms to the lower respiratory tract and avoids problems associated with uneven recovery from anesthesia among the animals. The small animal ventilator has been adapted for mice by appropriate setting changes made by the vendor.

For "nose only" delivery of aerosol, a Lovelace nebulizer was initially used to generate an aerosol of liposomes. Evidence of some denaturation of proteins and sheer force damage to liposomes was demonstrated. A modified DeVilbiss Aerosonic ultrasonic nebulizer in line with a multiport "nose only" aerosol chamber was thereafter used, and there has been no evidence of liposome damage by this method. Aerosol particle size distribution as measured by mass median diameter (MMD) for liposome aerosols was assayed using a Mercer-type 7 stage cascade impactor. ^{99m}Tc-DPTA was used to determine whether a delivery method results in diffuse labeling of the lungs. Furthermore, an "evenness index" can be easily calculated when fluorescently labeled agents are used. In particular, evenness of distribution for agents encapsulated in liposomes can be assayed by FITC labeled the agent and determination of the

resulting fluorescent pattern (CytoFluor and confocal microscopy). To enhance liposome delivery to murine lung, liposomes were prepared with anti-IgG conjugated to TRITC, then nebulized and delivered to mice using the multiport aerosol chamber for 30 min. The mice were then sacrificed, and AMs were obtained by BAL and examined by differential interference contrast and epifluorescence microscopy.

The inventor's model results in generation of liposome aerosols from the nebulizer with an MMD of 4.2 μm and from the "nose-only" port of 3.8 μm which is in the midrange of particle size for alveolar deposition. The aerosol results in diffuse labeling of both lungs (FIG. 5C). After only 30 min of nebulization, fluorescently labeled liposomes can already be detected inside AMs.

TRITC-labeled *M. avium* (20×10^6) were transthoracically injected into the upper portion of the right lung. An ultrasonic aerosol of FITC-liposomes was given for 30 min, and the right upper lobe was examined by confocal microscopy. The confocal micrograph reveals colocalization of TRITC-*M. avium* and FITC-labeled liposomes in the same alveolar spaces following 30 min of nebulization. This clearly demonstrates the potential feasibility of using aerosol to target liposomes to a localized mycobacterial infection in the lung. Since AMs will phagocytose both *M. avium* and liposomes, the putative therapeutic agents can be specifically delivered to the target cells in the alveolar spaces harboring the organisms.

EXAMPLE 7

EFFICACY TESTING OF NEW THERAPIES IN MYCOBACTERIAL PNEUMONIA

Two different approaches are used in the modeling of mycobacterial pulmonary infections. First, I.T. instillation of mycobacteria as recently described (Emori *et al.*, 1994) is used to induce a diffuse bilateral pneumonia. Second, since many types of mycobacterial pneumonias are localized and unilateral, the inventors have adapted a method used in pigs (Stine *et al.*, 1991) to induce a localized pneumonia using transthoracic administration of the mycobacteria. The localized pneumonia model was developed to test the hypothesis that aerosolization can achieve "colocalization" of the therapeutic agent with the mycobacteria in the isolated portion of the lung. Depending on the experimental design, inoculum may vary from 10 to 100×10^6 mycobacteria. Both methods work well in the BALB/c, SCID and CD4

lymphocyte-depleted mice. However, immunocompetent BALB/c mice appear resistant to I.T. *M. avium* (25 or 50×10^6) with no evidence of abnormal histopathology at 30 days after injection. CD4 lymphocyte-depleted mice and SCID mice show rapid evolution of pneumonia during the 30 days after I.T. delivery with mortality at approximately 30%.

5 The location of the injected material *via* the transthoracic method (30 g needle) can be verified by use of iodinated dye and fluoroscopic guidance and/or a mammographic unit. Controls receive transthoracic injections of vehicle alone and have no discernible injury 30 days after injection. Histopathology in SCID mice of the unaffected and the affected lungs are shown 14 days after transthoracic injection.

10 Clear endpoints such as mycobacterial viability, survival and histopathologic changes may be monitored. The "chest X-ray" of the mouse shows localization of the injected material in the upper portion of the right lung. Whereas the right lower and left lung are normal, the right upper lung specimen shows mycobacterial pneumonia teeming with *M. avium* organisms. The transthoracic model is not only useful for demonstrating a localized pneumonia, but is
15 applicable for studies of mycobacterial pneumonia in each of the models: BALB/c, CD4 lymphocyte-depleted and SCID mice.

* * *

20 All of the methods and compositions disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and compositions and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically,
25 it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

30

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

5

Anderson, *et al.*, "Effects on experimental acute lung injury 24 hours after exogenous surfactant instillation", *Exp. Lung. Res.*:18:191-204, 1992.

Anfossi, *et al.*, "An oligomer complementary to *c-myc* encoded mRNA inhibits proliferation of human myeloid leukemia cells lines." *Proc. Natl. Acad. Sci. USA*, 86:3379-3383, 1989.

10

Azghani, *et al.*, "A beta-linked mannan inhibits adherence of *Pseudomonas aeruginosa* to human lung epithelial cells." *Glycobiology*, 5:39-44, 1995.

Azghani, *et al.*, "Interaction of *Pseudomonas aeruginosa* with human lung fibroblasts: role of bacterial elastase." *Am. J. Cell Mol. Biol.*, 6:652-657, 1992.

15

Baker *et al.*, "Suppression of human colorectal carcinoma cell growth by wild-type *p53*," *Science*, 249:912-915, 1990.

Baker, "Adhesins and receptors of *Pseudomonas aeruginosa* associated with infection of the respiratory tract," *Microb. Pathol.*, 13:251-260, 1992.

Baker, *et al.*, "*Pseudomonas aeruginosa*, exoenzyme S is an adhesin." *Infect. Immun.*, 59(9):2859-2863, 1991.

20

Bakewell, *et al.*, "Confocal laser scanning immunofluorescence microscopy of lamellar bodies and pulmonary surfactant A in isolated alveolar type II cells." *Lab. Invest.*, 65:87-95, 1991.

Bangham, *et al.*, "Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.*, 13:(1)238-252, 1965.

25

Bangham, *et al.*, "The action of steroids and streptolysin S on the permeability of phospholipid structures to cations". *J. Mol. Biol.*, 13:(1)253-259, 1965.

Bischoff, *et al.*, "Introduction of 5'-terminal functional groups into synthetic oligonucleotides for selective immobilization". Department of Biochemistry, Purdue University, West Lafayette, Indiana. *Anal Biochem* 164:(2)336-344, 1987.

30

Bloom and Murray, "Tuberculosis: commentary on a reemergent killer". *Science*: 257:1055-1053, 1992.

Buchi *et al.*, *Cell Structure and Function* 18:399-407, 1993.

- Cacalano, *et al.*, "Production of the *Pseudomonas aeruginosa* neuraminidase in increased under hyperosmolar conditions and is regulated by genes involved in alginate expression." *J. Clin. Invest.*, 89:1866-1874, 1992.
- Canetti, G., "Exogenous reinfection and pulmonary tuberculosis. A study of the pathology." *Tuber. Lung. Dis.* 31:224-233, 1950.
- Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 76:425, 1977.
- Carnoy, *et al.*, "*Pseudomonas aeruginosa* outer membrane adhesions for human respiratory mucus glycoproteins," *Infect. Immun.*, 62(5):1896-1900, 1994.
- Caruthers, *et al.*, "Synthesis and biological studies of dithioate DNA." *Ciba Foundation Symposium.* 158:158-166, 1991.
- Centers for Disease Control and Prevention, "Initial therapy for tuberculosis in the era of multidrug resistance: recommendations of the advisory council for the elimination of tuberculosis". *JAMA* 273:220-226, 1995.
- Collins, "Cystic fibrosis: molecular biology and therapeutic implications." *Science.* 256:774-779, 1992.
- Connolly and Rider, "Chemical synthesis of oligonucleotides containing a free sulphhydryl group and subsequent attachment of thiol specific probes", *Nucleic Acids Res.* 13:(12)4485-4502, 1985.
- Connolly, B. A., "The synthesis of oligonucleotides containing a primary amino group at the 5'-terminus", *Nucleic Acids Res.* 15:(7)3131-3139, 1987.
- Cosstick and Eckstein, "Synthesis of d(GC) and d(CG) octamers containing alternating phosphorothioate linkages: effect of the phosphorothioate group on the B-Z transition." *Biochemistry*, 24(14):3630-3638, 1985.
- Davis, "Pathophysiology of pulmonary disease in cystic fibrosis." *Semin. Respir. Infect.*, 6(4):261-270, 1985.
- Doudorf and Palleroni, "Genus I. *Pseudomonas aeruginosa*." In: *The Bergey's Manual of determinative bacteriology*, 8th ed., Buchanan RE and Gibbons NE (ed.), The Williams and Wilkins Co., Baltimore, p. 217-243.
- Eckstein, "Oligonucleotide and analogues: a practical approach." IRL Press Oxford, 1991.
- Eckhardt and Luhrmann, "Blocking of the initiation of protein biosynthesis by a pentanucleotide complementary to the 3'-end of *Escherichia coli* 16S rRNA," *J. Biol. Chem.*, 254(22):11185-11188, 1979.

Editorial. "Pulmonary infection in cystic fibrosis -- still a sticky problem." *Lancet*, 1372-1373, 1986.

Emori. *et al.*, "Development of experimental model of animals for disseminated Mycobacterium avium complex infections using immunodeficient mice and rats". *Kekkaku* 69:317-322, 1994.

Ezekowitz. *et al.*, "Uptake of *Pneumocystis carinii* mediated by macrophage mannose receptor," *Nature*, 351:155-158, 1991.

Farschtschi and Gorenstein, "Preparation of a deoxynucleoside thiophosphoramidite intermediate in the synthesis of nucleoside of phosphorodithioates." *Tetrahedron Lett.* 29(52):6843-6846, 1988.

Gait, "An introduction to modern methods of DNA synthesis, in *Oligonucleotide synthesis: A Practical Approach*. Gait, M. J. Ed., IRL Press Oxford, 1984.

Ghosh and Bachhawat, "Targeting of liposomes to hepatocytes." In: Wu G, Wu C ed. *Liver diseases, targeted diagnosis and therapy using specific receptors and ligands*. New York: Marcel Dekker, pp. 87-104, 1991.

Gilligan, "Microbiology of airway disease in patients with cystic fibrosis," *Clin. Microbiol. Rev.* 4:35-51, 1991.

Gilman. *et al.*, Pharmacological Basis of Therapeutic, pp. 1018-1164, 1990.

Gould and Wise, "*Pseudomonas aeruginosa*: clinical manifestations and management," *Lancet*, pp. 1224-1226, 1985.

Govan, "Alginate biosynthesis and other unusual characteristics associated with pathogenesis of *Pseudomonas aeruginosa* in cystic fibrosis," In: *Bacterial infections of respiratory and gastrointestinal mucosae*, Griffiths, Donachie, and Stephen (ed), IRL Press Oxford, pp 67-96, 1988.

Gregoriadis. *et al.*, Drug Carriers in Biology and Medicine, pp. 287-341, 1979.

Grimwood. *et al.*, "Inhibition of *Pseudomonas aeruginosa* exoenzyme expression by subinhibitory antibiotic concentrations," *Antimicrob. Agents Chemother.*, 31:41-47, 1989a.

Grimwood. *et al.*, "Subinhibitory antibiotics reduce *Pseudomonas aeruginosa* tissue injury in the rat lung model," *J. Antimicrob. Chemother.*, 24:937-45, 1989b.

Hagenbuchle *et al.*, *Cell*, 13, 551, 1978.

- Harris, *et al.* "Effect of exogenous surfactant instillation on experimental acute lung injury". *J Appl Physiol* 66:2846-1851, 1989.
- Heikkila, *et al.*, "A *c-myc* antisense oligodeoxynucleotide inhibits enter into S phase but do not progress from G0 to G1." *Nature*, 328:445-449, 1987.
- 5 Hooper and Wolfson. "Mode of action of the quinolone antimicrobial agents: review of the recent information," *Rev. Infect. Dis.*, 11(Suppl. 5):902-11, 1989.
- Horn, *et al.*, Low. "Biotin-mediated delivery of exogenous macromolecules into soybean cells." *Plant Physiol.*, 93:1942-1496, 1992.
- Hutchin and Cortopassi. "Proposed molecular and cellular mechanism for aminoglycoside ototoxicity." *Antimicrobial Agents and Chemotherapy*, 38(11):2517-2520, 1994.
- 10 Iyer, *et al.*, "3H-1,2-Benzodithiole-3-one 1,1-dioxide as an improved sulfurizing reagent in the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioates. *J. Am. Chem. Soc.* 112:1253-1254, 1990.
- Jayaraman, *et al.*, "Selective inhibition of *Escherichia coli* protein synthesis and growth by nonionic oligonucleotides complementary to the 3'-end of 16S rRNA," *Proc. Natl. Acad. Sci. USA*, 78(3):1537-1541, 1981.
- 15 Jensen, *et al.*, "Nutritional factors controlling exocellular protease production by *Pseudomonas aeruginosa*," *J. Bacteriol.*, 144:844-847, 1980.
- Jiang, *et al.*, "Altered fluid transport across epithelium in cystic fibrosis," *Science* 262:424-427, 1993.
- 20 Johanson, Woods, Coauthor, "Association of respiratory tract colonization with adherence of gram-negative bacilli cells," *J. Infect. Dis.*, 139:667-673, 1979.
- Kaneda *et al.*, "Increased expression of DNA cointroduced with nuclear protein in adult rat liver", *Science*, 243:375-378, 1989.
- 25 Kato *et al.*, "Expression of hepatitis β virus surface antigen in adult rat liver," *J. Biol. Chem.*, 266:3361-3364, 1991.
- Kerem, *et al.*, "Pulmonary function and clinical course in patients with cystic fibrosis after pulmonary colonization with *Pseudomonas aeruginosa*," *J. Pediatr.*, 116:714-719, 1990.
- 30 Kharazmi, *et al.*, "Effect of *Pseudomonas aeruginosa* proteases on human leukocyte phagocytosis and bactericidal activity." *Acta Pathol. Microbiol. Immunol. Scand. (Sect. C)*, 94:175-179, 1986.

- Kharazmi, *et al.*, "Interaction of *Pseudomonas aeruginosa* alkaline protease and elastase with human polymorphonuclear leukocytes *in vitro*." *Infect. Immun.*, 43(1):161-165, 1984a.
- Kharazmi, *et al.*, "*Pseudomonas aeruginosa* exoproteases inhibit human neutrophil chemiluminescence." *Infect. Immun.*, 44(3):587-591, 1984b.
- 5 LaPlanche, *et al.*, "Phosphorothioate-modified oligodeoxyribonucleotides. III. NMR and UV spectroscopic studies of the Rp-Rp, Sp-Sp, and Rp-Sp duplexes. [d(GGSAATTCC)]₂, derived from diastereomeric O-ethyl phosphorothioates." *Nucleic Acids Res.*, 14(22):9081-9093, 1986.
- Leamon and Low, "Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis." *Proc. Natl. Acad. Sci. USA*, 88:5522-5526, 1991.
- 10 Lechner and LaVeck, "A serum-free methods for culturing normal human bronchial epithelial cells at clonal density," *J. Tissue Culture Meth.*, 9(2):43-48, 1985.
- Limper and Martin II, "*Pneumocystis carinii*: inhibition of lung cell growth mediated by parasite attachment." *J. Clin. Invest.*, 85:391-396, 1990.
- 15 Lin and Prusoff, "A novel synthesis and biological activity of several 5-halo-5'-amino analogues of deoxyribopyrimidine nucleosides", *J. Med. Chem.* 21:(1)106-109, 1978.
- Lin and Prusoff, "Synthesis and biological activity of several amino analogues of thymidine", *J Med Chem* 21:(1)109-112, 1978.
- Lloyd-Still and Wessel, "Advances and controversies in cystic fibrosis." *Semin. Respir. Infect.*, 20 11(3):197-210, 1990.
- Maniatis, *et al.*, *Molecular cloning: A laboratory manual*, p. 68, 1989.
- Martin II and Taylor, "Abnormal interaction of alpha₁ antitrypsin and leukocyte elastolytic activity in patients with chronic obstructive pulmonary disease," *Am. Rev. Respir. Dis.*, 120:482-486, 1979.
- 25 Maturka, *et al.*, "Regulation of viral expression by an antisense phosphorothioate oligodeoxynucleotide against rev(art/trs) in chronically infected cells." *Proc. Natl. Acad. Sci. USA*, 86:4244-4248, 1989.
- Mendelson, *et al.*, "*Pseudomonas aeruginosa* bacteremia in patients with AIDS." *Clin. Infect. Dis.*, 18:886-895, 1994.
- 30 Miller, *et al.*, "Solid-Phase Syntheses of oligodeoxyribonucleoside methylphosphonates," *Biochemistry*, 25:5092-5097, 1986.

- Miller, *et al.*, "Biochemical and biological effects of nonionic nucleic acid methylphosphonates." *Biochemistry*, 20:1874-1880, 1980.
- Myers, *et al.*, "The synthesis of 4-methyl-umbelliferyl α -ketoside of N-acetylneuraminic acid and its use in a fluorometric assay for neuraminidase." *Anal. Biochem.*, 101:166-174, 1980.
- 5 Nardell, E., "Beyond Four Drugs, Public health policy and the treatment of the individual patient with tuberculosis", *Am. Rev. Respir. Dis.*, 148:2-5, 1993.
- Natarajan and Schmid, "Inositol phospholipid hydrolysis by rat sciatic nerve phospholipase C." *J. Of Neurochem.*, 49(6):1879-1887, 1987.
- 10 Neu, "Quinolone antimicrobial agents," *Ann. Rev. Med.*, 43:465-86, 1992.
- Nichols, *et al.*, "Bacterial infections in the acquired immune deficiency syndrome." *Am. J. Clin. Pathol.*, 92:787-790, 1989.
- Nielson, "DNA analogues with nonphosphodiester backbones." *Annu. Rev. Biophys. Biomol. Struct.*, 24:167-183, 1995.
- 15 Nielson, *et al.*, "Synthesis of dinucleotide phosphorodithioates via thioamidities." *Tetrahedron Lett.*, 29(43):5517-5520, 1988.
- Nishino and Powers, "*Pseudomonas aeruginosa* elastase. Development of a new substrate, inhibitors, and an affinity ligand," *J. Biol. Chem.*, 255(8):3482-3486, 1980.
- Ostroff and Vasil, "Identification of a new phospholipase C by analysis of an insertional mutation in the hemolytic phospholipase C structural gene of *Pseudomonas*." *J. Bacteriol.*, 196:4597-4601, 1987.
- 20 Ostroff, *et al.*, "Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa*," *J. Bacteriol.*, 172(10):5915-5923, 1990.
- Ostroff, *et al.*, "Mutation in the hemolytic phospholipase C operon results in decreased virulence of *Pseudomonas aeruginosa* PAOI grown under phosphate-limiting conditions." *Infect. Immun.*, 57:1369-1373, 1989.
- 25 Pedersen and Kharazmi, "Inhibition of human natural killer cell activity by *Pseudomonas aeruginosa* alkaline protease and elastase." *Infect. Immun.*, 55(7):986-989, 1987.
- Pottratz and Martin II, "Role of fibronectin in *Pneumocystis carinii* attachment to cultured lung cells." *J. Clin. Invest.*, 85(2):351-356, 1990.
- 30

- Powers, *et al.*, "Specificity of porcine elastase, human leukocyte elastase and cathepsin G. Inhibition with peptide chloromethyl ketones." *Biochim. Biophys. Acta.*, 485(1):156-166, 1997.
- Powis, "Inhibitors of phosphatidylinositol signaling as antiproliferative agents." *Cancer and Metastasis Reviews*, 12(1):91-103, 1994.
- 5 Prigozy *et al.*, *Immunity* 6:187-197, 1997.
- Prince, A., "Adhesins and receptors of *Pseudomonas aeruginosa* associated with infection of the respiratory tract". *Microb. Pathol.* 13:251-260, 1992.
- Protratz and Martin II, "Mechanism of *Pneumocystis carinii* attachment to cultured rat alveolar macrophages" *J Clin Invest* 86:1678-1683, 1990.
- 10 Protratz, *et al.*, "*Pneumocystis carinii* attachment to cultured lung cells by *Pneumocystis* gp120, a fibronectin binding protein". *J Clin Invest* 88:403-407, 1991.
- Ramphal, *et al.*, "Adherence of *Pseudomonas* to tracheal cells injured by influenza infection or by endotracheal intubation," *Infect. Immun.*, 27(2):614-619, 1980.
- 15 Raviglione, *et al.*, "Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic". *JAMA* 273:270-226, 1996.
- Reddy, *et al.*, "Rapid assessment of mycobacterial growth inside macrophages and mice, using the radiometric (BACTEC) method", *Tuber. Lung. Dis.* 75:127-131, 1994.
- Reichman, L.B., "The U-shaped curve of concern", *Am. Rev. Respir. Dis.* 144:741-742, 1991.
- 20 Rolston and Bodey, "*Pseudomonas aeruginosa* infection in cancer patients." *Cancer Invest.*, 10:43-59, 1992.
- Saiman, *et al.*, "The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* respiratory epithelial monolayers." *J. Infect. Dis.*, 161:541-548, 1990.
- 25 Sanders, *et al.*, "Selection of multiple antibiotic resistance by quinolones, β -lactams, and aminoglycosides with special reference to cross-resistance between unrelated drug classes." *Antimicrob. Agents Chemother.*, 26:797-801, 1984.
- Sauer, *et al.*, "DNA and RNA virus species are inhibited by xanthates, a class of antiviral compounds with unique properties," *Proc. Natl. Acad. Sci. USA*, 81(11):3263-3267, 1984.
- 30

- Schwocho, *et al.*. "Cloning and characterization of a 3-*N*-aminoglycoside acetyltransferase gene, *aac(6)-Ib*, from *Pseudomonas aeruginosa*," *Antimicrobial Agents and Chemotherapy*, 39(8):1790-1796, 1995.
- Shaw, *et al.*. "Modified deoxyoligonucleotides stable to exonuclease degradation in serum," *Nucleic Acids Res.*, 19:747-750, 1991.
- Shine and Dalgarno. "The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites", *Proc. Natl. Acad. Sci. USA* 71:1342-13346, 1974.
- Shotton. "Confocal scanning microscopy and its applications for biological specimens." *J. Cell Sci.*, 94:175-206, 1989.
- Simpson, *et al.*. "Characterization of *Pseudomonas aeruginosa* *fliO*, a gene involved in flagellar biosynthesis and adherence." *Infect. Immun.*, 63:2950-2957, 1995.
- Simpson, *et al.*. "Genetic Analysis of *Pseudomonas aeruginosa* adherence: distinct genetic loci control attachment to epithelial cells and mucins." *Infect. Immun.*, 60:3771-3779, 1992.
- Stec, *et al.*, "Reversed-phase high-performance liquid chromatographic separation of diastereomeric phosphorothioate and analogues of oligodeoxyridonucleotides and other backbone-modified congeners of DNA," *J. Chromatogr.*, 326:263-280, 1985.
- Stein, *et al.*. "Physiochemical properties of phosphorothioate oligonucleotides," *Nucl. Acids Res.*, 16:3209-3221, 1988.
- Stine, *et al.*. "*Actinobacillus pleuropneumoniae*-induced thymic lesions in mice and pigs", *Infect Immun* 59:2885-2891, 1991.
- Szoka and Papahadjopoulos, "Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation", *Proc. Natl. Acad. Sci.*, 75:(9) 4194-4198, 1978.
- Taniguchi and Weissmann. "Inhibition of Q β RNA 70s ribosome initiation complex formation by an oligonucleotide complementary to the 3'-terminal region of *E. coli* 16S ribosomal RNA." *Nature*, 27:770-772, 1978.
- Theander, *et al.*. "Inhibition of human lymphocyte proliferation and cleavage of interleukin-2 by *Pseudomonas aeruginosa* proteases," *Infect. Immun.*, 56(7):1673-1677, 1988.
- Toren, *et al.*. "Determination of impurities in nucleoside 3'-phosphoramidites by fast atom bombardment mass spectrometry", *Anal. Biochem.* 152:(2)291-294, 1986.

- Toschka, *et al.*, "Complete nucleotide sequence of a 16S ribosomal RNA gene from *Pseudomonas aeruginosa*," *Nucleic Acids Res.*, 16(5):2348, 1988.
- Trudel, *et al.*, "Effect of oligonucleotide AGAGGAGGU on protein synthesis *in vitro*," *Biochimie*, 63:235-240, 1981.
- 5 Uster and Deamer, "pH-dependent fusion of liposomes using titratable polycations", *Biochemistry* 24:(1) p1-8, 1985.
- van der Giessen, *et al.*, "Comparison of the 23S ribosomal RNA genes and the spacer region between the 16S and 23S rRNA genes of the closely related *Mycobacterium avium* and *Mycobacterium paratuberculosis* and the fast-growing *Mycobacterium phlei*",
- 10 *Microbiology* 140:1103-1108, 1994.
- Vasil, "Pseudomonas aeruginosa: biology, mechanism of virulence, and epidemiology," *J. Pediatr.*, 108(2):800-805, 1986.
- Wachter, *et al.*, "A simple and efficient procedure for the synthesis of 5'-aminoalkyl oligodeoxynucleotides", *Nucleic Acids Res.* 14:(20)7985-7994, 1986.
- 15 Winnie and Cowan, "Respiratory tract colonization with *Pseudomonas aeruginosa* in cystic fibrosis: correlations between anti-*Pseudomonas aeruginosa* antibody levels and pulmonary function," *Pediatr. Pulmonol.*, 10:92-100, 1991.
- Wisniowski and Martin II, "Interaction of vitronectin with *Pneumocystis carinii*: evidence for binding *via* the heparin binding domain," *J. Lab. Clin. Med.*, 125(1):38-45, 1995.
- 20 Wisniowski, *et al.*, "Isolation of *Pneumocystis carinii* gp120 by fibronectin affinity: evidence for manganese dependence," *Am. J. Respir. Cell Mol. Biol.*, 11:262-269, 1994.
- Woods, *et al.*, "Pseudomonas aeruginosa exoenzyme S as a pathogenic determinant in respiratory infections," *Antibiotic Chemother.*, 42:27-35, 1986.
- Woods, *et al.*, "Role of fibronectin in the prevention of adherence of *Pseudomonas aeruginosa*
- 25 to buccal cells," *J. Infect. Dis.*, 143(6):784-790, 1981.
- Yosida, *et al.*, "Transforming growth factor-beta stimulates the expression of desmosomal proteins in bronchia epithelial cells," *Am. J. Of Respir. Cell and Mol. Biol.*, 6(4):439-445, 1992.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: ADVANCED RESEARCH & TECHNOLOGY INSTITUTE
- (B) STREET: 501 North Morton Street
- (C) CITY: Bloomington
- (D) STATE: IN
- (E) COUNTRY: USA
- (F) POSTAL CODE (ZIP): 47404

(ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR INHIBITING
BACTERIAL GROWTH

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 60/027,729
- (B) FILING DATE: 01-OCT-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGCCAACCTA GTGGAGGAAA G

21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCGGTTGGAT CACCTCCTTT C

21

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCGGUUGGAU CACCUCCUUU C

21

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CGCCAACCTA GTGGAGGAAA G

21

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGCCGACCTA GTGGAGGAAT

20

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCGGCTGGAT CACCTCCTTA

20

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGGTGACCCT CGTGAGAGAC

20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1537 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAACTGAAGA GTTTGATCAT GGCTCAGATT GAACGCTGGC AGCAGGGGCC TTCAACACAT	60
GCAAGTCGAG CTTATGAAGG GAGCTTGCCT TGGATTCAGC GGC GGACGGG TGAGTAATGC	120
CTAGGAATCT GCCTGGTAGT GGGGGATAAC GTCCGGAAAC GGCCGCTAAT ACCGCATACG	180
TCCTGAGGGA GAAAGTCGGG GATCTTCGGA CCTCACGCTA TCAGATGAGC CTAGGTCGGA	240
TTAGCTAGTT GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCGTAACTGG TCTGAGAGGA	300
TGATCAGTCA CACTGGA ACT GAGACACGGT CCAGACTCCT ACGGGAGGCA GCAGTGGGGA	360
ATATTGGACA ATGGGCGCAA GCCTGATCCA GCCATGCCGC GTGTGTGAAG AAGGTCTTCG	420
GATTGTAAAG CACTTTAAGT TGGGAGGAAG GGCAGTAAGT TAATACCTTG CTGTTTGACG	480
TTACCAACAG AATAAGCACC GGCTAACTTC GTGCCAGCAG CCGCGGTAAT ACGAAGGGTG	540
CAAGCGTTAA TCGGAATTAC TGGGCGTAAA GCGCGCGTAA GTGGTTCAGC AAGCTTGATG	600
TGAAATCCCC GGGCTCAACC TGGGAACTGC ATCCAAAAGC TACTGAGCTA GAGTACGGTA	660
GAGGTGGTAG AATTTCTGT GTAGCGGTGA AATGCGTAGA TATAGGAAGG AACACCAAGTG	720
GCGAAGGCGA CCACCTGGAC TGTACTGACA CTGAGGTGCG AAAGCGTGGG GAGCAAACAG	780
GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGTCGA CTAGCCGTTG GGATCCTTGA	840
GATCTTAGTG GCGCACGTAA CGCGATAAGT CGACCGCCTG GGGAGTACGG CCGCAAGGTT	900
AAA ACTCAAA TGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT TTAATTCGAA	960
GCAACGCGAA GAACCTTACC TGGCCTTGAC ATGCTGAGAA CTTTCCAGAG ATGGATTGGT	1020
GCCTTCGGGA ACAGAGACAC AGGTGCTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT	1080
TGGGTAAAGT CCCGTAACGA GCGCAACCCT TGTCTTAGT TACCAGCACC TCGGGTGGGC	1140
ACTCTAAGGA GACTGCCGGT GACAAACCGG AGGAAGGTGG GGATGACGTC AAGTCATCAT	1200
GGCCCTTACG GCCAGGGCTA CACACGTGCT ACAATGGTCG GTACAAAGGG TTGCCAAGCC	1260

GCGAGTGGGA GCTAATCCCA TAAAACCGAT CGTAGTCCGG ATCGCAGTCT GCAACTCGAC 1320
 TGCCTGAAGT CGGAATCGCT AGTAATCGTG AATCAGAATG TCACGGTGAA TACGTCCCCG 1380
 GGCCTTGTAC ACACCGCCCC TCACACCATG GGAGTGGGTT GCTCCAGAAG TAGCTAGTCT 1440
 AACCGCAAGG GGGACGGTTA CCACGGAGTG ATTCATGACT GGGGTGAAGT CGTAACAAGG 1500
 TAGCCGTAGG GGAACCTGCG GCTGGATCAC CTCCTTA 1537

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 317 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GATCACCTCC TTTCTAAGGA GCACCACGAA AAGCACTCCA ATTGGTGGAG TGCAAGCCGT 60
 GAGGGGTTCT CGTCTGTAGT GGACGAAAAC CGGGTGCACA ACAGCAAAT GATTGCCAGA 120
 CACACTATTG GGCCCTGAGA CAACACTCGG TCGAACCGTG TGGAGTCCCT CCATCTTGGT 180
 GGTGGGGTGT GGTGTTTGAG TATTGGATAG TGGTTGCGAG CATCTAGATG AACGCGTGGT 240
 CCTTCGTGGC CGGCGTGTTT ATCGAAATGT GTAATTTCTT CTTTGGTTTT TGTGTGTAAG 300
 TAAGTGTTTA AGGGCGC 317

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1271 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GTTGCCGACG GCGACACCGA GGTCCACGAT GTATTCCACA TCGATCGCGG ATATCCGTTG 60
 TTCGTGGAGA ACCTGGTGAG TCTCGGTGCC GAGATCGAAC GGGTATGCTG TTAGGCGACG 120
 GTCACCTATG GATATCTATG GATGACCGAA CCTGGTCTTG ACTCCATTGC CGGATTTGTA 180
 TTAGACTGGC AGGGTTGCCC CGAAGCGGGC GGAAACAAGC AAGCGTGTTG TTTGAGAACT 240
 CAATAGTGTG TTTGGTGGTT TCACATTTTT GTTGTTATTT TTGGCCATGC TCTTGATGCC 300
 CCGTTGTCGG GGGCGTGGCC GTTTGTTTTG TCAGGATATT TCTAAATACC TTTGGCTCCC 360
 TTTTCCAAAG GGAGTGTTTG GGTTTTGTTC GGAGAGTTTG ATCCTGGCTC AGGACGAACG 420

```

CTGGCGGCGT GCTTAACACA TGCAAGTCGA ACGGAAAGGT CTCTTCGGAG ATACTCGAGT      480
GGCGAACGGG TGAGTAACAC GTGGGTGATC TGCCCTGCAC TTCGGGATAA GCCTGGGAAA      540
CTGGGTCTAA TACCGGATAG GACCACGGGA TGCATGTCTT GTGGTGGAAA GCGCTTTAGC      600
GGTGTGGGAT GAGCCCGCGG CCTATCAGCT TGTGTGGTGGG GTGACGGCCT ACCAAGGCGA      660
CGACGGGTAG CCGGCCTGAG AAGGTGTCCG GCCACACTGG GACTGAGATA CGGCCCAAAC      720
TCCTACGGGA GGCAGCAGTG GGAATATTG CACAATGGGC GCAAGCCTGA TGCAGCGACG      780
CCGCGTGGGG GATGACGGCC TTCGGGTTGT AAACCTCTTT CACCATCGAC GAAGGTCCGG      840
GTTCTCTCGG ATTGACGGTA GGTGGAGAAG AAGCACCGGC CAACTACGTG CCAGCAGCCG      900
CGGTAATACG TAGGGTGCGA GCGTTGTCCG GAATTACTGG GCGTAAAGAG CTCGTAGGTG      960
GTTTGTGCGG TTGTTGCTGA AATCTCACGG CTTAACTGTG AGCGTGCGGG CGATACGGGC     1020
AGACTAGAGT ACTGCAGGGG AGACTGGAAT TCCTGGTGTA GCGGTGGAAT GCGCAGATAT     1080
CAGGAGGAAC ACCGGTGGCG AAGGCGGGTC TCTGGGCAGT AACTGACGCT GAGGAGCGAA     1140
AGCGTGGGGA GCGAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GGTGGGTACT     1200
AGGTGTGGGT TTCCTTCCTT GGGATCCGTG CCGTAGCTAA CGCATTAAAGT ACCCCGCCTG     1260
GGGAGTACGG C                                                    1271

```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1555 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```

TTTTATGGAG AGTTTGATCC TGGCTCAGGA TGAACGCTGG CGGCGTGCCT AATACATGCA      60
AGTCGAGCGA ACGGACGAGA AGCTTGCTTC TCTGATGTTA GCGGCGGACG GGTGAGTAAC      120
ACGTGGATAA CCTACCTATA AGACTGGGAT AACTTCGGGA AACCGGAGCT AATACCGGAT      180
AATATTTTGA ACCGCATGGT TCAAAAGTGA AAGACGGTCT TGCTGTCACT TATAGATGGA      240
TCCGCGCTGC ATTAGCTAGT TGGTAAGGTA ACGGCTTACC AAGGCAACGA TACGTAGCCG      300
ACCTGAGAGG GTGATCGGCC AACTGGAAC TGAGACACGG TCCAGACTCC TACGGGAGGC      360
AGCAGTAGGG AATCTTCCGC AATGGGCGAA AGCCTGACGG AGCAACGCCG CGTGAGTGAT      420
GAAGGTCTTC GGATCGTAAA ACTCTGTTAT TAGGGAAGAA CATATGTGTA AGTAACTGTG      480

```

CACATCTTGA CGGTACCTAA TCAGAAAGCC ACGGCTAACT ACGTGCCAGC AGCCGCGGTA 540
ATACGTAGGT GGCAAGCGTT ATCCGGAATT ATTGGGCGTA AAGCGCGCGT AGGCGGTTTT 600
TTAAGTCTGA TGTGAAAGCC CACGGCTCAA CCGTGGAGGG TCATTGGAAA CTGGAAAAGT 660
TGAGTGCAGA AGAGGAAAGT GGAATTCCAT GTGTAGCGGT GAAATGCGCA GAGATATGGA 720
GGAACACCAG TGGCGAAGGC GACTTTCTGG TCTGTAAGT ACGCTGATGT GCGAAAGCGT 780
GGGGATCAAA CAGGATTAGA TACCCTGGTA GTCCACGCCG TAAACGATGA GTGCTAAGTG 840
TTAGGGGGTT TCCGCCCTT AGTGCTGCAG CTAACGCATT AAGCACTCCG CCTGGGGAGT 900
ACGACCGCAA GGTGAAAGT CAAAGGAATT GACGGGGACC CGCACAAGCG GTGGAGCATG 960
TGGTTTAATT CGAAGCAACG CGAAGAACCT TACCAAATCT TGACATCCTT TGACAACTCT 1020
AGAGATAGAG CCTTCCCCTT CGGGGGACAA AGTGACAGGT GGTGCATGGT TGTCGTCAGC 1080
TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC AACCTTAAG CTTAGTTGCC 1140
ATCATTAAGT TGGGCACTCT AAGTTGACTG CCGGTGACAA ACCGGAGGAA GGTGGGGATG 1200
ACGTCAAATC ATCATGCCCC TTATGATTG GGCTACACAC GTGCTACAAT GGACAATACA 1260
AAGGGCAGCG AAACCGCGAG GTCAAGCAAA TCCCATAAAG TTGTTCTCAG TTCGGATTGT 1320
AGTCTGCAAC TCGACTACAT GAAGCTGGAA TCGCTAGTAA TCGTAGATCA GCATGCTACG 1380
GTGAATACGT TCCCGGGTAT TGTACACACC GCCCGTCACA CCACGAGAGT TTGTAACACC 1440
CGAAGCCGGT GGAGTAACCT TTTAGGAGCT AGCCGTCGAA GGTGGGACAA ATGATTGGGG 1500
TGAAGTCGTA ACAAGGTAGC CGTATCGGAA GGTGCGGCTG GATCACCTCC TTTCT 1555

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1535 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TAAACTGAAG AGTTTGATCC TGGCTCAGAT TGAACGCTGG CGGCATGCTT AACACATGCA 60
AGTCGAACGG CAGCACGGGT GCTTGCACCT GGTGGCGAGT GGCGAACGGG TGAGTAATAC 120
ATCGGAACAT GTCCTGTAGT GGGGGATAGC CCGGCGAAAG CCGGATTAAT ACCGCATACG 180
ATCTACGGAT GAAAGCGGGG GACCTTCGGG CCTCGCGCTA TAGGGTTGGC GATGGCTGAT 240
TAGCTAGTTG GTGGGGTAAA GGCCTACCAA GGCGACGATC AGTAGCTGGT CTGAGAGGAC 300
GACCAGCCAC ACTGGGACTG AGACACGGCC CAGACTCCTA CGGGAGGCAG CAGTGGGGAA 360

TTTTGGACAA TGGGCGAAAAG CCTGATCCAG CAATGCCGCG TGTGTGAAGA AGGCCTTCGG	420
GTTGTAAAGC ACTTTTGTCC GGAAAGAAAT CCCTGGCTCT AATACAGTCG GGGGATGACG	480
GTACCGGAAG AATAAGCACC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT ACGTAGGGTG	540
CAAGCGTTAA TCGGAATTAC TGGGCGTAAA GCGTGCGCAG GCGGTTTGCT AAGACCGATG	600
TGAAATCCCC GGGCTCAACC TGGGAACTGC ATTGGTGA CT GGCAGGCTAG AGTATGGCAG	660
AGGGGGGTAG AATTCCACGT GTAGCAGTGA AATGCGTAGA GATGTGGAGG AATACCGATG	720
GCGAAGGCAG CCCCCTGGGC CAATACTGAC GCTCATGCAC GAAAGCGTGG GGAGCAAACA	780
GGATTAGATA CCCTGGTAGT CCACGCCCTA AACGATGTCA ACTAGTTGTT GGGGATTCAT	840
TTCCTTAGTA ACGTAGCTAA CGCGTGAAGT TGACCGCCTG GGGAGTACGG TCGCAAGATT	900
AAAAC TCAAA GGAATTGACG GGGACCCGCA CAAGCGGTGG ATGATGTGGA TTAATTCGAT	960
GCAACGCGAA AAACCTTACC TACCCTTGAC ATGGTCGGAA TCCTGCTGAG AGGTGGGAGT	1020
GCTCGAAAGA GAACCGGCGC ACAGGTGCTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT	1080
GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTTGTCCTTA GTTGCTACGC AAGAGCACTC	1140
TAAGGAGACT GCCGGTGACA AACCGGAGGA AGGTGGGGAT GACGTCAAGT CCTCATGGCC	1200
CTTATGGGTA GGGCTTCACA CGTCATACAA TGGTCGGAAC AGAGGGTTGC CAACCCGCGA	1260
GGGGGAGCTA ATCCCAGAAA ACCCATCGTA GTCCGGATTG CACTCTGCAA CTCGAGTGCA	1320
TGAAGCTGGA ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG TTCCCGGGTC	1380
TTGTACACAC CGCCCGTCAC ACCATGGGAG TGGGTTTTAC CAGAAGTGGC TAGTCTAACC	1440
GCAAGGAGGA CGGTCACCAC GGTAGGATTC ATGACTGGGG TGAAGTCGTA ACAAGGTAGC	1500
CGTATCGGAA GGTGCGGCTG GATCACCTCC TTTCT	1535

CLAIMS

1. A method for inhibiting bacterial protein expression comprising the steps of:

- 5 (i) providing an oligonucleotide of between about 10 to about 35 consecutive bases of the 3'-end of a bacterial 16S rRNA; and
- (ii) contacting said oligonucleotide with a bacterium,

whereby protein expression of said bacterium is inhibited.

10

2. The method of claim 1, wherein said oligonucleotide is modified to include a transport moiety.

3. The method of claim 1, wherein said oligonucleotide is formulated in a liposome.

15

4. The method of claim 1, wherein said bacterium is selected from the group consisting *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Moraxella lacunata*, *Streptomyces scabies*, *C. perfringens*, *E.coli*, *Salmonella typhi*, *Cornebacterium coyleiae*, *Magnetic coccus*, *Azoarcus evansii*, *Sphingomonas trueperi*, *Burkholderia*, and *Chlamydia*.

20

5. The method of claim 2, wherein said transport moiety is selected from the group consisting of an amino acid, biotin, folate and a carbohydrate.

25 6. The method of claim 5, wherein said transport moiety is selected from the group consisting of an amino acid, biotin, folate and a carbohydrate.

7. The method of claim 6, wherein said amino acid is selected from the group consisting of arginine, asparagine, glutamine, glycine, valine, isoleucine, leucine, methionine, proline, tryptophan and valine.

30

8. The method of claim 1, wherein said oligonucleotide comprises the sequence C-C-U/T-C-C.
9. The method of claim 1, wherein said oligonucleotide comprises at least one phosphoramidite residue.
10. The method of claim 3, wherein said liposome further comprises a bacterial targeting moiety.
11. A method for treating a bacterial infection in a patient comprising the steps of:
- (i) providing a liposomal formulation comprising an oligonucleotide of between about 10 to about 35 consecutive bases of the 3'-end of a bacterial 16S rRNA in a pharmaceutically acceptable form; and
 - (ii) administering said liposomal formulation to said patient.
12. The method of claim 11, wherein said oligonucleotide is modified to include a transport moiety.
13. The method of claim 11, further comprising administering to said patient an antibiotic.
14. The method of claim 13, wherein said antibiotic is a sulfonamide, a quinolone, a penicillin, a cephalosporin, a beta-lactam antibiotic, an aminoglycoside, or a tetracycline.
15. An oligonucleotide of between about 10 to about 35 consecutive bases of the 3'-end of a bacterial 16S rRNA.
16. The oligonucleotide of claim 15, wherein said 16S rRNA is derived from *P. aeruginosa*, *M. tuberculosis*, *M. avium*.

17. The oligonucleotide of claim 15, wherein said oligonucleotide is modified to include a transport moiety.

18. The oligonucleotide of claim 15, wherein said transport moiety is selected from the group consisting of an amino acid, biotin, folate and a carbohydrate.

19. The oligonucleotide of claim 18, wherein said amino acid is selected from the group consisting of arginine, asparagine, glutamine, glycine, valine, isoleucine, leucine, methionine, proline, tryptophan and valine.

20. The oligonucleotide of claim 15, wherein said oligonucleotide comprises the sequence C-C-U/T-C-C.

21. The oligonucleotide of claim 15, wherein said oligonucleotide comprises at least one phosphoramidite residue.

22. The oligonucleotide of claim 15, wherein said oligonucleotide is about 15 bases in length.

23. The oligonucleotide of claim 15, wherein said oligonucleotide is about 20 bases in length.

24. The oligonucleotide of claim 15, wherein said oligonucleotide is about 25 bases in length.

25. The oligonucleotide of claim 15, wherein said oligonucleotide is about 25 bases in length.

26. The oligonucleotide of claim 15, wherein said oligonucleotide is selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12.

27. A liposomal formulation comprising an oligonucleotide of between about 10 to about 35 consecutive bases of the 3'-end of a bacterial 16S rRNA.
- 5 28. The liposomal formulation of claim 27, wherein liposomes of the formulation include multilamellar vesicles.
29. The liposomal formulation of claim 27, wherein said liposomes comprise at least one of the lipids selected from the group consisting of DMPC, DCP, DMPG and cholesterol.
- 10 30. The liposomal formulation of claim 27, wherein liposomes of said formulation further comprise a bacterial targeting moiety.

1/5

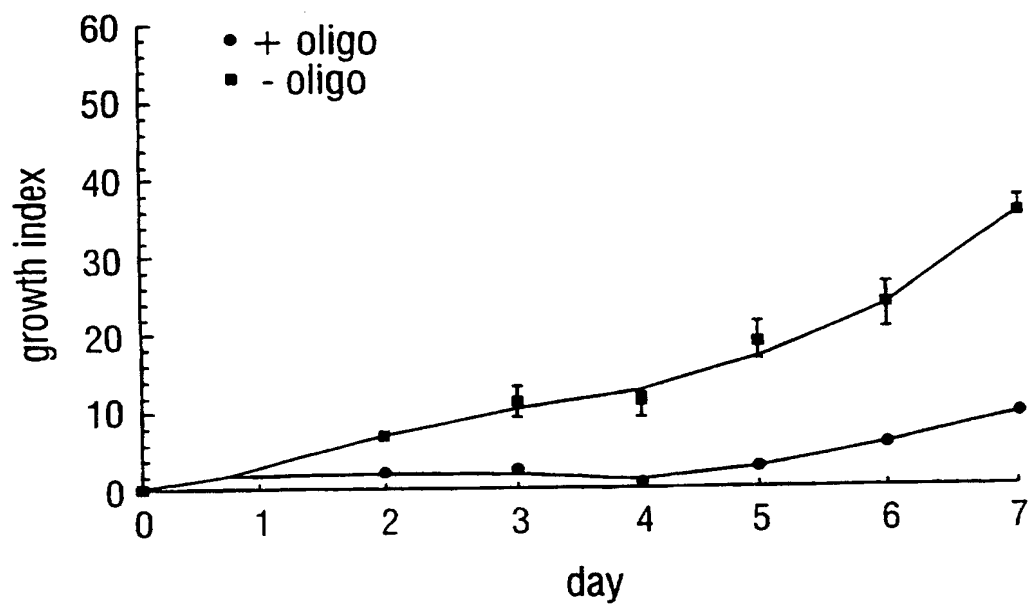


FIG. 1A

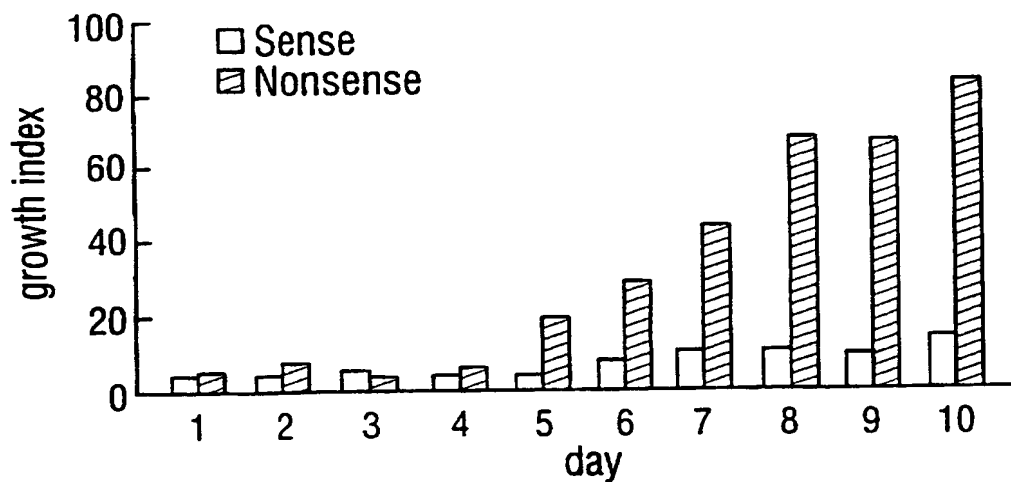


FIG. 1B

SUBSTITUTE SHEET (RULE 26)

2/5

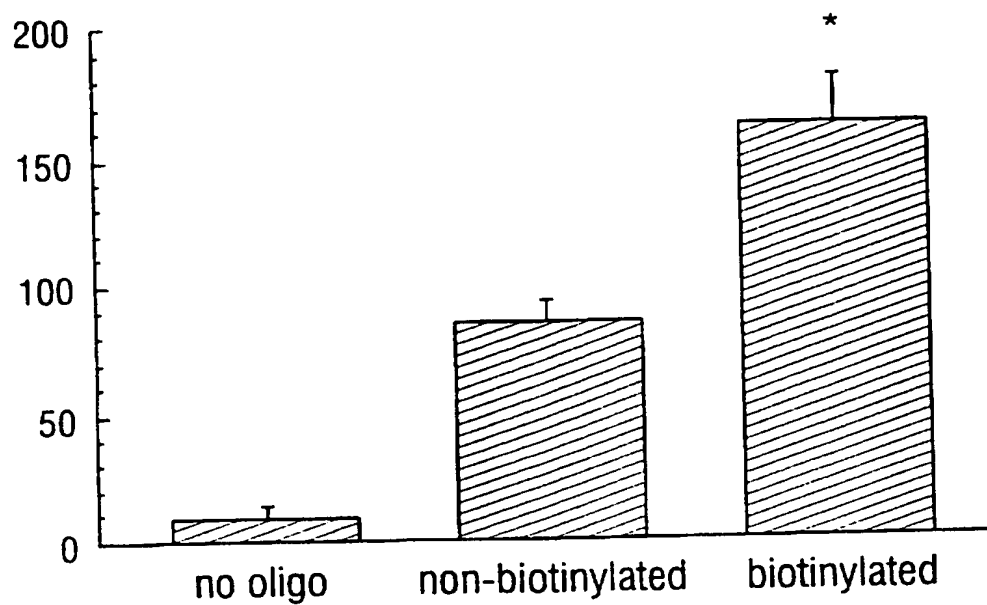


FIG. 2A

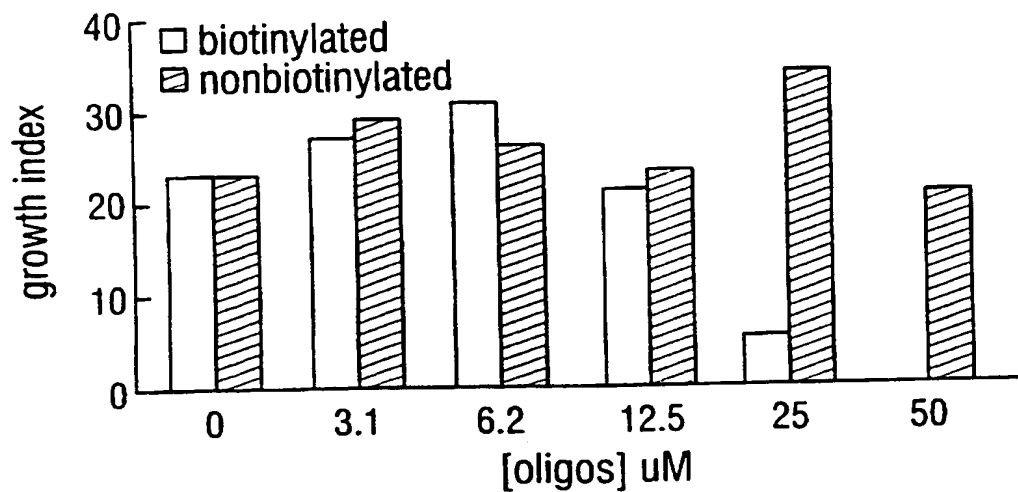


FIG. 2B

SUBSTITUTE SHEET (RULE 26)

3/5

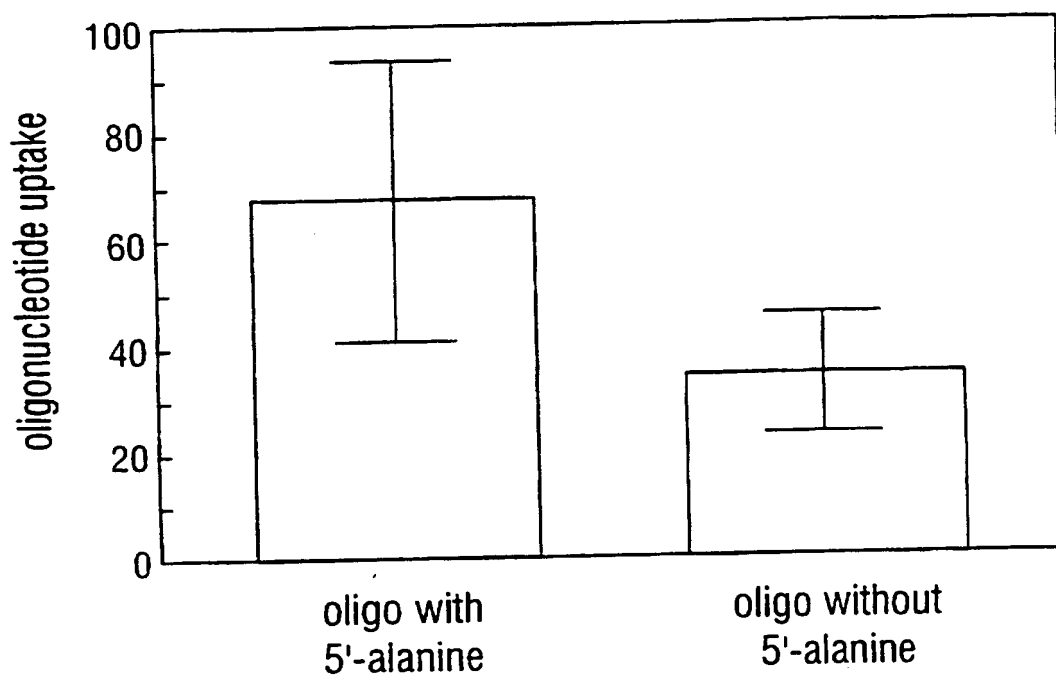


FIG. 3

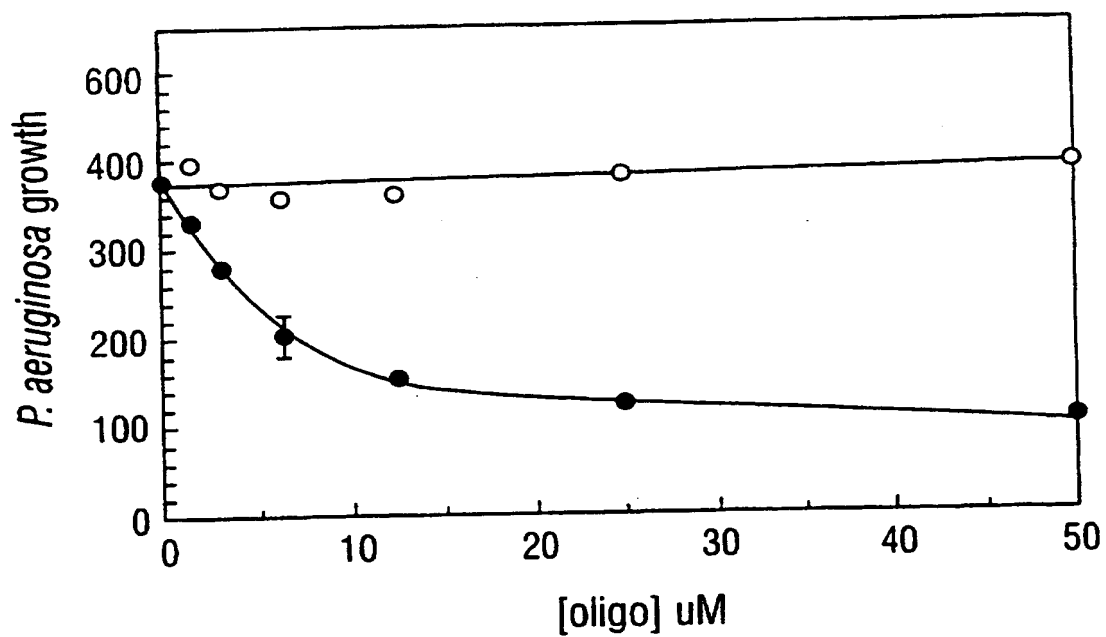


FIG. 4

SUBSTITUTE SHEET (RULE 26)

4/5

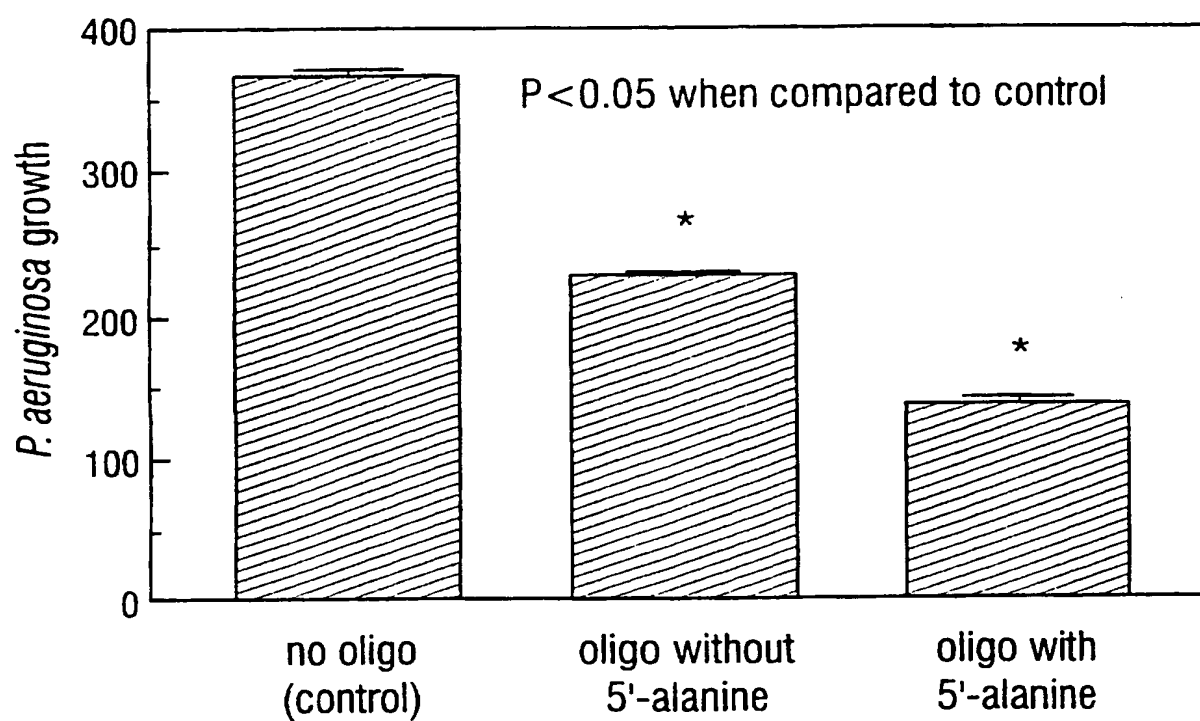


FIG. 5

5/5

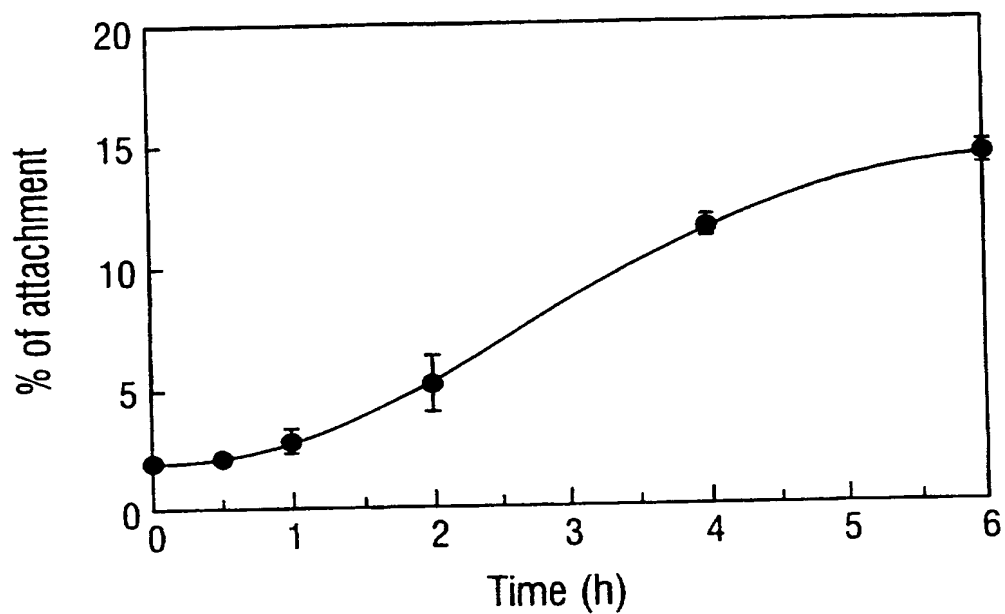


FIG. 6A

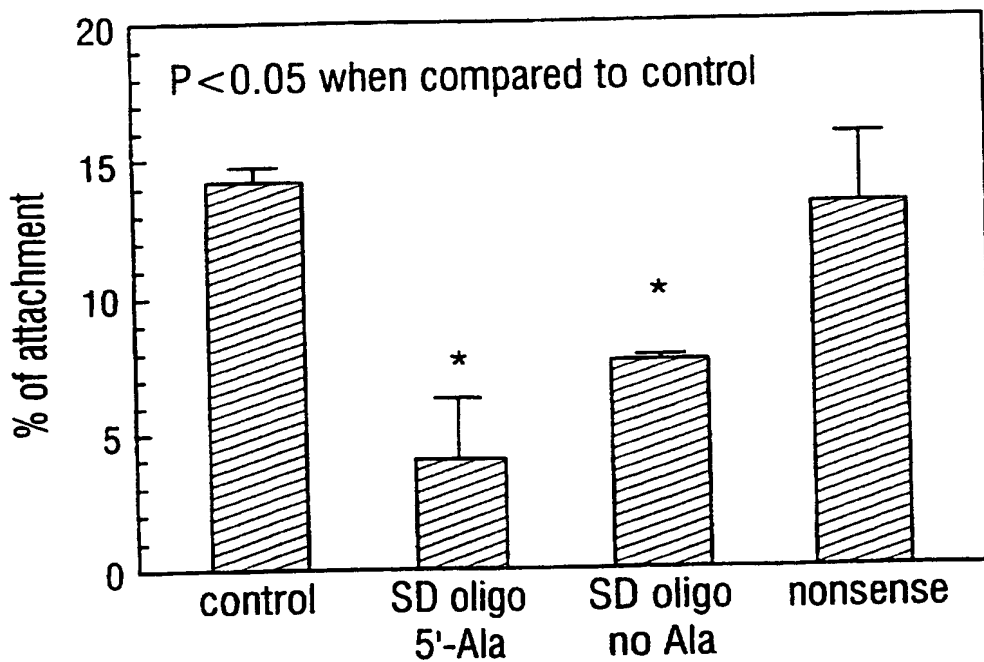


FIG. 6B

SUBSTITUTE SHEET (RULE 26)



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/11, C07H 21/00, A61K 31/70, 47/22	A3	(11) International Publication Number: WO 98/14567 (43) International Publication Date: 9 April 1998 (09.04.98)
(21) International Application Number: PCT/US97/18094 (22) International Filing Date: 30 September 1997 (30.09.97) (30) Priority Data: 60/027,729 1 October 1996 (01.10.96) US (71) Applicant (for all designated States except US): ADVANCED RESEARCH & TECHNOLOGY INSTITUTE [US/US]; 501 North Morton Street, Bloomington, IN 47404 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MARTIN, William, J. [US/US]; 7556 Chablis Circle, Indianapolis, IN 46278 (US). WISNIOWSKI, Paul [US/US]; 5914 Petersburg Parkway, Indianapolis, IN 46202 (US). (74) Agent: HIGHLANDER, Steven, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 16 July 1998 (16.07.98)
(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING BACTERIAL GROWTH (57) Abstract <p>The present invention relates generally to the field of bacteriology. More particularly, it concerns methods and compositions for the treatment of bacterial infection employing oligonucleotides targeted to the Shine-Dalgarno region of prokaryotes to inhibit bacterial protein expression and hence inhibit bacterial infection.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

Interr. Application No
PCT/US 97/18094

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/11 C07H21/00 A61K31/70 A61K47/22		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07H A61K C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 00624 A (BAYLOR COLLEGE MEDICINE) 25 January 1990	1,2, 5-10,15, 17-25
Y	see page 17, line 9 - page 19, line 4 see claims 22,23	2-7, 10-14, 16-19, 26-30
Y	--- TOSCHKA, H. ET AL.: "Complete nucleotide sequence of a 16S ribosomal RNA from Pseudomonas aeruginosa" NUCLEIC ACIDS RESEARCH., vol. 16, 1988, OXFORD GB, page 2348 XP002055330 cited in the application see the whole document --- <div style="text-align: right;">-/--</div>	4,16,26
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center; font-size: 1.2em;">12 February 1998</div>		Date of mailing of the international search report <div style="text-align: center; font-size: 1.2em;">27 -05- 1998</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center; font-size: 1.2em;">ANDRES S.M.</div>

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 97/18094

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 93 23570 A (PHARMAGENICS INC) 25 November 1993</p> <p>see page 4, line 11 - page 8, line 11 see page 17, line 19 - page 18 see page 23, line 6 - line 14 see example 5 see claims</p>	<p>2,3,5-7, 10-12, 17-19, 27-30</p>
Y	<p>WO 96 24378 A (WORCESTER FOUND EX BIOLOGY ;RAPAPORT ELIEZER (US); METELEV VALERI) 15 August 1996 see the whole document</p>	<p>2,5-7, 11-14</p>
X	<p>WO 95 27054 A (UNIV MONTREAL ;BRAKIER GINGRAS LEA (CA); MELANCON PIERRE (CA); COT) 12 October 1995 see page 6, line 25 - page 9</p>	<p>1,15,22</p>
X	<p>EP 0 140 308 A (UNIV NEW YORK) 8 May 1985 see page 20, line 7 - page 24 see page 27, line 5 - page 30 see page 38</p>	<p>1,15</p>
X	<p>RAHMAN M A ET AL: "ANTIBACTERIAL ACTIVITY AND INHIBITION OF PROTEIN SYNTHESIS IN ESCHERICHIA COLI BY ANTISENSE DNA ANALOGS" ANTISENSE RESEARCH AND DEVELOPMENT, vol. 1, 1991, pages 319-327, XP002045888 see oligonucleotide 1533 and figure 1</p>	<p>1,15</p>
A	<p>THIERRY A R ET AL: "LIPOSOMAL DELIVERY AS A NEW APPROACH TO TRANSPORT ANTISENSE OLIGONUCLEOTIDES" GENE REGULATION, BIOLOGY OF ANTISENSE RNA AND DNA, 1992, ERICKSON R P;IZANT J G, pages 147-161, XP002040368 see the whole document</p>	<p>3,11, 27-30</p>
A	<p>TANIGUCHI, T. & WEISSMANN, C.: "Inhibition of Qbeta RNA 70S ribosome initiation complex formation by an oligonucleotide complementary to the 3' terminal region of E.coli 16S ribosomal RNA" NATURE., vol. 275, 26 October 1978, LONDON GB, pages 770-772, XP002055331 cited in the application</p>	

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/18094

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>JAYARAMAN K ET AL: "SELECTIVE INHIBITION OF ESCHERICHIA COLI PROTEIN SYNTHESIS AND GROWTH BY NONIONIC OLIGONUCLEOTIDES COMPLEMENTARY TO THE 3' END OF 16S RRNA" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 78, no. 3, March 1981, pages 1537-1541, XP002045889 cited in the application</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

I. International application No.

PCT/US 97/ 18094

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see annex
2. ☐ Claims Nos.:
because they relate to parts of the international Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See annex

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-30 (all partially) (see subject 1)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-30 (all partially)

A method for inhibiting *Pseudomonas aeruginosa* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

2. Claims: 1-30 (all partially)

A method for inhibiting *Mycobacterium* spp. protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

3. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting *Moraxella lacunata* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

4. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting *Streptomyces scabies* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

5. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting *C. perfringens* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

6. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting *E. coli* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

7. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting *Salmonella typhi* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

8. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting *C. coyleia* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

9. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting *Magnetic coccus* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

10. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting *Azoarcus evansii* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

11. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting *Sphingomonas trueperi* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

12. Claims: 1-15,17-30 (all partially)

A method for inhibiting *Burkholderia* protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

13. Claims: 1-15,17-25,27-30 (all partially)

A method for inhibiting Chlamydia protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

14. Claims: 1-3,5-15,17-30 (all partially)

A method for inhibiting S. aureus protein expression with oligonucleotides interfering with the ribosomal binding of mRNAs, conjugates of the oligonucleotides and pharmaceutical compositions containing them.

Remark : Although claims 1 to 10 (as far as in vivo methods are concerned) and claims 11 to 14 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/18094

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9000624 A	25-01-90	AT 137806 T AU 4180889 A DE 68926455 D DE 68926455 T EP 0424473 A JP 3505672 T US 5294533 A	15-05-96 05-02-90 13-06-96 31-10-96 02-05-91 12-12-91 15-03-94
WO 9323570 A	25-11-93	NONE	
WO 9624378 A	15-08-96	AU 5259796 A CA 2212682 A	27-08-96 15-08-96
WO 9527054 A	12-10-95	AU 2064495 A CA 2186107 A EP 0753063 A JP 9511142 T US 5707866 A	23-10-95 12-10-95 15-01-97 11-11-97 13-01-98
EP 0140308 A	08-05-85	DE 3486053 A EP 0467349 A JP 2694924 B JP 9135686 A JP 2651442 B JP 60232092 A US 5190931 A US 5272065 A US 5208149 A	11-03-93 22-01-92 24-12-97 27-05-97 10-09-97 18-11-85 02-03-93 21-12-93 04-05-93



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/11, C07H 21/00, A61K 31/70, 47/22	A3	(11) International Publication Number: WO 98/14567 (43) International Publication Date: 9 April 1998 (09.04.98)
(21) International Application Number: PCT/US97/18094 (22) International Filing Date: 30 September 1997 (30.09.97) (30) Priority Data: 60/027,729 1 October 1996 (01.10.96) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/027,729 (CIP) Filed on 1 October 1996 (01.10.96) (71) Applicant (for all designated States except US): ADVANCED RESEARCH & TECHNOLOGY INSTITUTE [US/US]; 501 North Morton Street, Bloomington, IN 47404 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MARTIN, William, J. [US/US]; 7556 Chablis Circle, Indianapolis, IN 46278 (US). WISNIOWSKI, Paul [US/US]; 5914 Petersburg Parkway, Indianapolis, IN 46202 (US). (74) Agent: HIGHLANDER, Steven, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 16 July 1998 (16.07.98)
(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING BACTERIAL GROWTH (57) Abstract The present invention relates generally to the field of bacteriology. More particularly, it concerns methods and compositions for the treatment of bacterial infection employing oligonucleotides targeted to the Shine-Dalgarno region of prokaryotes to inhibit bacterial protein expression and hence inhibit bacterial infection.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)